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UNIVERSITY OF ALBERTA

MOLECULAR AND CELLULAR BASES OF THE ROLE OF GROWTH HORMONE IN BOVINE MAMMARY BIOLOGY

BY

DAVID R. GLIMM

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

ANIMAL BIOCHEMISTRY

DEPARTMENT OF ANIMAL SCIENCE

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Molecular and Cellular Bases of the Role of Growth Hormone in Bovine Mammary Biology

SUBMITTED BY David R. Glimm

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy in Animal Biochemistry.

J.J. Kennelly

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J.D. Turner

Date: October 12, 1990

To

the memory of my father, Helmuth Glimm.

His love and encouragement will be remembered always.

To

the memory of my best friend ever, Freddie.

To

my wife, Janice, whose love and patience makes my efforts bearable.

ABSTRACT

Injection of lactating dairy cows with growth hormone (GH) results in a marked increase in milk production. This response clearly indicates that the action of GH is important in the regulation of mammary cell physiology. It is the objective of this thesis to provide insight into the role of GH from this perspective. The possibilities of both direct and indirect GH actions on mammary tissue were investigated.

GH receptor mRNA transcripts were identified in mammary tissue and their abundance was lower in GH-treated cows. This suggests that GH receptor down regulation may occur in mammary tissue during GH treatment, and be regulated at the level of transcription or mRNA turnover. It was shown that the GH receptor gene is expressed primarily in the alveolar epithelial cells of mammary tissue. These results suggest that the lactating bovine mammary gland is a GH target tissue. They also challenge the widely accepted view that GH does not directly regulate mammary growth or function.

An analysis of serum insulin-like growth factor-I (IGF-I) revealed that GH injection of lactating dairy cows induces greater than a two-fold increase in serum IGF-I concentration. This finding suggests that IGF-I may play a role in inducing the lactation response during GH treatment. IGF-I, type I IGF receptor, and epidermal growth factor (EGF) receptor mRNAs were studied to examine the possibility of indirect GH action on mammary processes. Several IGF-I transcripts were identified in lactating mammary tissue; GH treatment resulted in a decrease in the largest (7.4 kb) IGF-I transcript. This finding may reflect a GH-directed increase in IGF-I synthesis in mammary tissue. Type I IGF receptor mRNA was also identified; the type I receptor gene was predominantly expressed in epithelial cells. An EGF receptor mRNA was detected and shown to be most abundant in epithelial cells. The abundance of all type I IGF and EGF receptor transcripts was lower in mammary tissue from GH-treated cows. These findings may reflect receptor down regulation and therefore implicate IGF-I and EGF action in the regulation of epithelial cell physiology during GH treatment. Collectively, these results establish alterations in the biology of several signaling molecules that are potentially relevant to mammary changes induced by GH injection.

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INTRODUCTION

Without exception, the mammary gland and the milk it produces during lactation are essential to the survival of neonates from the over 4,000 different species of mammals (43). Even milk formulas used for human neonates rely on milk obtained from the mammary gland of cows. The mammary gland of agricultural species serves a dual purpose because it not only contributes to neonatal survival, but also provides a source of raw milk used to manufacture milk and milk products for human consumption.

Underlying the quest for knowledge about mammary and lactation physiology is the fundamental desire to understand the complexities of these biological events. However, there is also a need to develop new strategies to increase the efficiency of milk production in agricultural species, as well as a need for more effective diagnostic and treatment procedures in the management of human breast diseases. Continued progress toward each of these goals is dependent upon research that increases our understanding of mammary biology in general.

Collectively, knowledge gained through mammary research using agricultural animals, humans, and laboratory animals has provided a framework to facilitate progress toward a better understanding of mammary physiology. Our current understanding is indeed far from complete. Nevertheless, it is known that the same cell types are present in mammary tissue of all mammals (113). Fundamental processes of mammary physiology, including mammary cell interactions, are also similar in all mammals (70, 175, 23). Where apparent differences exist, they are often understandable in terms of differences in reproductive strategies (38, 56, 82). The reason for the difference in the incidence of mammary tumors between humans and dairy cows is a notable exception, which remains elusive (154). Learning more about the details of mammary biology will ultimately lead to further progress in the dairy industry, as well as improvements in the management of human breast disease.

This thesis is an account of observations on growth hormone (GH) biology made in the course of studies concerned with the control of mammary function. The impetus

for this research came from earlier studies, which demonstrated the remarkable ability of endogenously administered pituitary extracts to stimulate lactation in cows (7, 35, 54, 76, 192). Although the first of these studies was conducted over fifty years ago, relatively little progress has been made toward understanding the physiological basis of this phenomenon. However, there has recently been renewed interest in this area; advances in recombinant DNA methodology have created a limitless source of GH (58, 95, 136, 147, 149).

The production and health effects of long-term GH injection of dairy cattle are now the subject of intensive investigation. By contrast, considerably less research effort has been devoted to understanding the molecular and cellular bases of the lactation response to GH injection. It is the objective of this thesis to provide insight into the role of GH from this perspective. Accordingly, an overview of general mammary development and function is presented to provide a conceptual framework for understanding how the actions of GH may contribute to the control of mammary processes. The experimental strategy employed is based on inference from this understanding. The objective is to substantiate suppositions about the role of GH in mammary processes.

MAMMARY DEVELOPMENT

Development of the mammary gland begins during embryogenesis and has been extensively characterized, particularly in the mouse (5, 139, 173). Early in mouse embryogenesis, in both the female and male embryo, cells from the ectodermal zone migrate to, and descend into the dermis to form mammary buds on either side of the ventral midline. Under the influence of the surrounding mesenchyme mammary development continues, but only in the female fetus. In the male fetus the onset of androgen production by the testes apparently halts further mammary bud development (96).

During the last several days of female fetal life, a rapid proliferation of mammary epithelial cells takes place and gives rise to the mammary cord. Shortly

before term, the mammary cord opens to the exterior as the future nipple. At around this stage the cord also becomes partially canalized and begins to branch at its distal end.

Although inhibition of mammary rudiment growth in the male fetus seems critical for sexual dimorphism in mammary development, endocrine control of development in the female fetus is poorly understood. However, it is well established that mesenchymal influences play a vital role in normal fetal mammary development (103, 150). Interactions between epithelial and stromal cells are also important at other stages of mammary development, as well as during lactation.

Although only limited mammary development occurs during juvenile life, periods of isometric and then allometric growth of the gland occur before puberty (156, 157, 163). During puberty there is extensive epithelial proliferation in the form of ductal growth. By the time sexual maturity is reached, the epithelium has extended throughout the mammary fat-pad. However, a region of fat is left devoid of ducts in anticipation of pregnancy, at which time it will become occupied by alveolar epithelial lobules.

There has been extensive research into hormonal control of pubertal mammary ductal growth and ductal maintenance (173). Some differences, or at least apparent differences, in the hormonal requirements to support ductal growth in various species have been demonstrated. Although some variability in endocrine control can be expected, it is also likely that at least some of the reported differences are due to differences in experimental systems and hormone preparations used to study ductal development. Nevertheless, a common pattern of hormonal control of duct development is evident. Estrogens are essential to normal ductal development. The results of both *in vivo* estrogen replacement therapy studies in ovariectomized animals and *in vitro* studies support this role for estrogen (92, 120). However, estrogens alone do not support ductal growth, but require the presence of prolactin (173). In addition to these requirements, it is apparent that as yet unknown stromal influences also play a critical role in normal ductal growth (2). Various other hormones and growth factors have also

been shown to influence ductal growth, but the exact nature of their roles *in vivo* is unknown (173).

Mammary development during pregnancy, commonly referred to as mammeogenesis, is characterized by proliferation of mammary epithelium and its differentiation toward functional phenotypic expression. When mammeogenesis is complete, the intraductal fat zone is occupied by alveolar lobules connected to the mammary ducts with an extensive network of branched ductules (173). Increased mammary vascularization also occurs in conjunction with lobuloalveolar development (83, 162).

Systemic endocrine control of mammeogenesis involves the actions of hormones from the ovaries, pituitary, thyroid, and the adrenal gland; estrogens, progesterone, prolactin, thyroid hormones, and adrenal steroids have all been shown to affect lobuloalveolar development (93, 173, 181). That some of these hormones are effective at only particular stages of pregnancy, and some have little or no effect unless influenced previously by the actions of others, reveals clearly the complexity of the endocrine events involved in mammeogenesis. The fetus also exerts control in mammeogenesis in many species, primarily through the actions of placental lactogen (30, 56, 168).

In addition to the many hormonal synergisms that regulate mammeogenesis, several growth factors have also been implicated in the proliferation and differentiation of mammary epithelium (45, 85, 181). The effects of epidermal growth factor (EGF) have been most extensively studied, and its role as a primary mitogenic stimulator of mammary epithelial cells is well established (77, 155, 164, 172). Besides its mitogenic effect, EGF also inhibits the functional differentiation of epithelial cells (164). Progesterone also exerts a repressive action on differentiation, particularly during early pregnancy (173). Insulin responsiveness is acquired by the epithelia early in pregnancy, and insulin is then required for terminal differentiation (174). Glucocorticoids also play an obligatory role in the differentiative process (173). Along with the many hormones and growth factors that have regulatory roles in mammeogenesis are stromal-epithelial

interactions, which direct both morphological and differentiative changes during pregnancy (70).

The greatest increase in differentiation of mammary epithelial cells occurs close to parturition. This process, commonly referred to as lactogenesis, corresponds to the appearance of the cellular elements specifically required for synthesis of milk components (122). In conjunction with the attempt to delineate the hormonal trigger of lactogenesis is the question of whether this cellular event is primarily due to the removal of negative influences or the result of stimulatory influences. Although this issue has not been definitively resolved, it is believed that release from progesterone inhibition is essential for the onset of lactation (97). By contrast, the periparturient increase in both prolactin and glucocorticoids is believed to provide a crucial positive stimulus to initiate events required for successful lactation (74).

It may be overly simplistic to expect that the trigger for lactogenesis involves the actions of only one or a few hormones. Considering the many metabolic and ultrastructural events, as well as the complexity of regulation required for copious milk synthesis and secretion, it is more likely that a complex combination of regulatory molecules interact to regulate cellular events of lactogenesis. Indeed, the functional differentiative potential of mammary epithelial cells is already realized early in embryogenesis (31, 173). Milk protein mRNA synthesis begins in the virgin animal (145). The synthesis of all major milk components occurrs throughout mammary development in pregnancy (173). Furthermore, it is becoming increasingly apparent that numerous hormones and growth factors play regulatory roles in the synthesis of milk components (9). Thus, different combinations of regulatory signals must operate in a coordinated manner at each different stage of mammary development, including lactogenesis.

When lactation commences the mammary epithelial cells are finally fully differentiated, and thus capable of synthesizing and secreting large quantities of milk components in response to specific hormonal stimulus. The most obvious cytological features of alveolar epithelial cells in the mature functional state are a well polarized

cellular arrangement and substantial hypertrophy (122, 133). Present in the basal and paranuclear area are an extensive rough endoplasmic reticulum and a well formed system of Golgi cisternae. Many mitochondria and free ribosomes are located throughout the cytoplasm. In addition to these features, characteristic of exocrine cells, the cytoplasm of lactating alveolar epithelial cells also contains numerous casein- and lactose-laden secretory vesicles and lipid droplets. Using various cytoskeletal disrupting agents, it has been shown that both intact microtubule and microfilament systems are required for intracellular transport of secretory vesicles and lipid droplets (124, 161). Release of secretory vesicles, lipid droplets, and their contents through the apical plasma membrane into the alveolar lumen involves primarily exocytotic mechanisms. The single layer of secretory epithelial cells lining the alveoli are surrounded by myoepithelial cells, arranged in a branch-like or basket pattern around each alveolus. These cells contract in response to oxytocin, causing alveoli to expel luminal contents into mammary ducts.

Regulation of mammary gland physiology during lactation involves a myriad of systemic and local influences (55, 74). In addition to direct hormonal influences, the supply of substrates required for milk component synthesis, and thus the factors that regulate this general process are often also considered regulatory influences related to mammary function during lactation. The principle of this supposition is questionable considering that altering the amount of substrate available to the mammary gland does not necessarily alter the amount of milk produced (39, 66, 129). That some multifunctional molecules regulate intermediary metabolism in non-mammary tissues, as well as similar or different processes in mammary tissue seems a more plausible explanation for this type of regulatory interdependency. The most important regulatory factors in mammary biology during lactation are therefore those directly or indirectly involved in controlling the function, growth, or maintenance of mammary cells. Although a regulatory relationship likely exists between changes in mammary cell physiology and metabolic adaptations required for substrate supply during lactation, it

is probably important only insofar as it ensures both biological events operate concurrently.

The control of mammary cell growth during lactation is poorly understood, but it is known that fully differentiated, lactating alveolar epithelial cells are responsive to mitogenic stimuli. Proliferation during lactation has been reported in goats, cattle, and rodents (83, 94, 195). Most proliferation during lactation occurs early in lactation. This general pattern suggests that lactational proliferation is important at least in terms of achieving peak lactation. However, the actual contributions made by new cells to established lactation can only be determined when better quantitative estimates of cell growth during each stage of lactation become available. Recognizing the potential importance of such contributions and identifying the factors that regulate proliferation during lactation has obvious implications for increasing milk yield in agricultural species.

Proliferative events during lactation are likely regulated by a similar combination of signals that regulate growth during mammeogenesis, although fetal and placental influences would not be present. The marked difference in intensity of proliferation in these two physiological states indicates that positive proliferative forces dominate in mammeogenesis, while negative proliferative forces dominate in lactation. The inhibitory influences in lactation may be similar to those that operate in the prepubertal mammary gland. So far, no inhibitory compounds have been identified. The hormonal stimuli responsible for alveolar epithelial cell proliferation during lactation also remain to be identified.

Evidence of local control of proliferation during lactation is provided by experiments that investigated the effects of unilateral milking frequency on mammary proliferation in goats (94). Increased unilateral milking frequency resulted in a significant growth response in only the gland that received extra milking. This finding suggests that local influences are at least partly responsible for growth regulation during lactation. Whether local influences include the actions of stimulatory factors, inhibitory factors, or both remains to be established.

Another important aspect of mammary cell physiology during lactation is cell maintenance or longevity. Cell death after peak lactation is believed to be primarily responsible for the progressive decline in milk production toward the end of lactation (94). Although some studies have claimed that cell maintenance can be prolonged by imposing specific hormonal regimens, it is not clear whether experimental treatments affected cell maintenance, growth, biosynthetic activity, or some combination of these events (94, 148, 166). The systemic and local influences affecting maintenance of terminally developed alveolar epithelial cells have yet to be thoroughly investigated.

Accumulated knowledge of fundamental molecular and cell biology presents an overwhelming array of cellular processes of obvious importance to the function of lactating mammary cells. Such processes range from the biochemistry and physiology of substrate uptake and milk component synthesis and secretion, to the regulation of genes expressed in the lactating mammary cell. In association with normal cell function are also the overriding proliferative and differentiative forces that maintain the functional integrity of existing cells and induce recruitment of new cells.

The physiology of the lactating mammary cell is controlled by a continuous and complex pattern of biological signals. Regulatory signals include those from other tissues as well as those from the cell's immediate surrounding environment. Although there is growing interest in mammary cell-cell interactions and the role of the extracellular matrix in mammary function, the majority of mammary function research has focused on the roles of nonmammary-derived regulatory signals.

SUBSTRATE UPTAKE BY EPITHELIAL CELLS

Our understanding of cytological processes involved in the uptake of substrates from the circulation by alveolar epithelia cells is essentially limited to knowledge about the transport systems common to most cells. Substrate uptake from the circulation is for the most part accomplished by proteinaceous transporters located in the basal plasma membrane of alveolar epithelial cells (18).

At least six different amino acid transport systems are used to internalize bloodborne amino acids (15, 112). A continuous supply of amino acids is required to

synthesize the major milk proteins, such as caseins and whey proteins. The regulation of amino acid transport into lactating cells has not been characterized, but it presumably involves at least some of the same hormonal and adaptive regulatory mechanisms identified for other cell types.

Of the lipids found in milk some are synthesized *de novo* in mammary epithelial cells, using different precursors such as glucose and acetate, while others are derived from different forms of bloodborne lipids such as chylomicrons and other phospholipids (46). Uptake of substrates for milk-fat synthesis therefore involves a number of different transport systems. There is little information about the factors that regulate the different lipid and lipid precursor transport mechanisms in lactating cells; however, there is evidence to support a role for prolactin in stimulating the activity of lipoprotein lipase in mammary capillary endothelial cells (111, 153). The level of activity of this enzyme reflects the capacity of the tissue to remove lipids from the blood. Prolactin supposedly also plays a role in mediating the decrease in lipoprotein lipase activity in adipose tissue during lactation (194). It is proposed that this change serves to direct lipids to the mammary gland for milk fat synthesis.

Glucose is utilized by lactating cells primarily for lactose synthesis and to lesser, albeit varying degrees for *de novo* fat synthesis, depending on species (141, 185). A high rate of carrier-mediated glucose transport is required to support lactose synthesis. Insulin is known to be a key regulator of carrier-mediated glucose transport in nonmammary tissues, but the importance of its role in this process in mammary tissue remains controversial (66, 74, 99, 135). Although glucose uptake by lactating mammary cells *in vitro* is not acutely stimulated by insulin, as it is in other cell types, insulin is required for mammary cells to maintain a high basal rate of glucose transport *in vitro* (135). The observed effect of insulin on mammary glucose transport *in vitro* may be due to its positive effect on differentiative maintenance (174).

MAMMARY FUNCTION

Regulation of Mammary Protein Synthesis and Function

Besides milk proteins, lactating alveolar cells also synthesize a large number of other proteins, which serve diverse roles in cellular function and maintenance. Such proteins include those involved in intermediary metabolism, milk component synthesis, receiving and transferring biological signals, as well as those with structural roles. Clearly, a large number of complex regulatory mechanisms must also exist to coordinate not only the synthesis of all the different proteins, but also their actions.

The synthesis and degradation of proteins that simply serve structural roles, as components of the cell and its many organelles, is precisely controlled. After synthesis and modification, these proteins are directed to the proper cellular location where they become part of a controlled replacement process (21). In a resting cell, this series of events could seemingly be controlled by a constitutive type of regulatory mechanism. In a lactating cell, however, these events are markedly enhanced and their precise coordination probably involves more complex regulatory mechanisms. With the exception of several studies into the role of certain cytoskeletal elements in vesicular transport (4, 121, 123, 124, 161), there has been no research into the biology of structural proteins in lactating cells.

By contrast, there has been considerable research effort directed at understanding the role of specific components of the extracellular matrix in mammary function (2, 62, 90, 101, 104, 183). The impetus for research in this area came from studies that showed isolated epithelial cells regained secretory activity when cultured on type I collagen gels (50). Alveolar epithelial cells synthesize and assemble a continuous, mammary-specific basal lamina which completely surrounds each alveolus (126). *In vitro* experiments have demonstrated the dependency of this process on hormones such as insulin, prolactin, and hydrocortisone (2).

It is well established that an important functional relationship exists between the epithelium and the extracellular matrix (2, 103, 150). The extracellular matrix plays a fundamental role in maintaining proper cell morphology and in modulating the

synthesis and secretion of several milk proteins. That other structural proteins will also prove to have dynamic roles in mammary biology seems inevitable. Cytoskeletal and cell junction proteins may prove important in this regard.

Marked alterations in intermediary metabolism take place in the epithelial cell during lactation, in order to meet the large increase in demand for energy. The majority of research on energy metabolism in the lactating cell has focused on identifying major substrates, and thus metabolic pathways used to generate the extra energy required for cell function during lactation (6, 160). As a result, a great deal is known about the biochemistry of substrate metabolism in the lactating cell. Largely unknown, however, are the regulatory signals involved in initiating and controlling the necessary metabolic changes. Nevertheless, it can be surmised that some combination of signaling molecules are probably important in this regard. An important part of regulation of epithelial cell metabolism during lactation is undoubtedly control of the expression of rate-limiting enzyme genes, as well as the synthesis, degradation and activity of their expressed proteins. Any of the so-called lactogenic hormones are attractive candidates for roles as primary regulators of energy metabolism in the lactating epithelial cell.

Even with our present state of knowledge of the molecular biology of the cell, it is difficult to comprehend the complexities that must be involved in receiving and integrating the large number of biological signals required to control the function of a lactating cell. Nevertheless, researchers have begun to study some of the molecules implicated in biological signal transduction. The most obvious are specific proteins, called receptors, that interact with signaling molecules to initiate a biological response in the target cell. Such signaling molecules include growth factors, steroid and protein hormones, as well as other chemical mediators. Thus, the ability of a cell to respond to a particular signaling molecule is dependent on the presence of a specific receptor protein. Most receptor proteins are located on the cell surface, intimately associated with the plasma membrane, where their interaction with signaling molecules takes place. In some instances this interaction occurs in the cytoplasm (128, 187). Mammary

epithelial cells possess numerous specific protein receptors of both the cell surface and intracellular type. While the existence of some of these specific receptors has been confirmed experimentally, the existence of others is implied largely through the results of *in vitro* studies where biological responses have been demonstrated.

The responsiveness of a target cell to a particular signaling molecule depends upon several different factors. Changing the number of receptors at the cell surface is the most obvious mechanism by which a cell alters its responsiveness to extracellular signals. This change can be accomplished simply by regulating the rate at which receptors are synthesized or degraded. Alternatively, this can be accomplished by controlling the number of receptors recruited to the cell surface from an intracellular receptor pool. Another mechanism involves inactivating or altering the ability of receptors to interact with chemical mediators. When other proteins are involved in transducing the biological signal after receptor activation, target cell responsiveness can be altered by changing the intracellular concentration or activity of the other proteins (125, 171, 190). A less obvious, but commonly observed mechanism involves the regulated recycling of receptors (187, 188). Almost invariably ligand-receptor binding at the cell surface triggers receptor-mediated endocytosis. After intracellular dissociation of the ligand-receptor complex, receptors can be differentially targeted for either degradation or replacement at the cell surface (17).

Knowledge about specific receptors on mammary epithelial cells, and in particular the events surrounding their regulation, is essential to our understanding of mammary development and function. So far, research into mammary receptors has concentrated on studying the receptors that specifically bind progesterone, estrogen, or prolactin (87, 117, 182, 184). Mammary estrogen receptor research has in fact contributed to the development of effective endocrine therapies in breast cancer treatment (152). These therapies generally involve treating breast cancer patients, which have specific symptoms including estrogen receptor-positive tumors, with antiestrogenic agents. Other mammary receptor research, notably that involving the EGF receptor and the closely related *erb*B-2 protein, has recently contributed exciting

information that may lead to a better understanding of mammary malignancy (91, 159, 191). Future research in this area holds promise for the development of more effective treatment strategies for breast cancer.

The majority of research on prolactin receptors in mammary tissue has studied their biology in the context of normal development or function, whereas fewer studies have investigated prolactin receptor biology in mammary carcinoma (87). Knowledge of the events underlying the ontogeny and regulation of prolactin receptors in mammary tissue is critical to understanding the exact role of prolactin in different mammary processes. Accumulated information indicates that the regulation of prolactin receptors is complex.

Prolactin receptor status has been studied during different physiological states and under various endocrine conditions. Numbers, but not affinity of prolactin receptors fluctuate according to the stage of pregnancy or lactation (49). Mammary prolactin receptor numbers generally increase during highly proliferative stages of mammary growth. Similarly, numbers generally increase around parturition when induction of mammary cell activity takes place.

In addition to prolactin playing a role in the regulation of its own receptor, inducing both up and down regulation, various other hormones have been shown to affect prolactin receptor status (182). As more is learned about the regulation of prolactin receptors, there will also be a better understanding of the role of prolactin in mammary biology. The recent cloning of the prolactin receptor gene provides an important tool that should facilitate progress in prolactin receptor research (26).

The key regulatory role played by receptors in most, if not all mammary processes makes them primary targets for research. Much remains to be learned about ontogeny and regulation of the many different receptors used by mammary epithelial cells to integrate signals in their environment. Future identification and characterization of as yet unknown signaling molecules, particularly those coupled to receptor-mediated events, will also contribute to a more complete understanding of the biology of the mammary epithelial cell.

Regulation of Milk Protein Synthesis

Milk protein synthesis is a principal function of mammary epithelial cells during lactation. In bovine milk, the major milk proteins are α_{s1}-, α_{s2}-, β-, and κ-casein, and the whey proteins, α-lactalbumin and β-lactoglobulin (115). The casein family of phosphoproteins represent about 80% of the total protein in bovine milk. The caseins provide a source of amino acids, calcium, and phosphorous to the neonatal animal. They have no known biological role in mammary epithelial cell function. On the other hand, α-lactalbumin, combined with galactosyltransferase plays an essential role in the biosynthesis of lactose (99). No specific biological role has been found for β-lactoglobulin, although it has been proposed that it may play a role in retinol transport (127).

The collective results from a large number of studies indicate that many different hormones, as well as other compounds are able to alter the overall rate of casein synthesis (9, 10, 28, 115, 140). Those reported include prolactin, glucocorticoids, progesterone, estrogen, thyroid hormones, insulin, and other compounds such as cholera toxin and phorbol myristate acetate, agents which activate protein kinase C. In addition, cell-cell interactions and the extracellular matrix are also believed to play a regulatory role in casein synthesis (2, 104). A number of hormones have also been implicated in the regulation of α-lactalbumin synthesis (137, 138), whereas the regulatory factors in β-lactoglobulin synthesis have not yet been identified.

Most recently, research on casein synthesis has involved analyses of the regulation of casein gene expression and casein mRNAs. These studies have been facilitated by the cloning of all the casein genes (2, 84, 104, 145, 167, 189). Similarly, the cloning of the α-lactalbumin gene has also facilitated analysis of its regulation (64, 75). The β-lactoglobulin gene has recently been cloned (80), but its regulation and corresponding mRNAs remain to be studied.

Considering the complexity of hormonal regulation of casein synthesis, it is not surprising that many regulatory events are also being uncovered at the nucleic acid level (167). It has been established that the hormonal induction of casein synthesis is

associated with enhanced casein mRNA accumulation. Furthermore, transcription rate changes, modulation of post-transcriptional processes, and alterations in casein mRNA stability are each, in part, responsible for the relationship between protein synthesis and mRNA accumulation (32). Specific basement membrane constituents are also involved in modulating casein gene expression (2, 104). In addition, there is evidence for hormonal regulation of casein mRNA translation (165).

Recent progress has also been made toward identifying the specific DNA sequences of the casein gene involved in regulating its expression (142). Future research will likely account for the effects of most casein synthesis regulatory factors identified to date. That as yet unknown regulatory factors will be identified also seems inevitable. So far, researchers have begun to delineate the roles of only prolactin, insulin, and glucocorticoid in casein gene transcription and mRNA kinetics.

Regulation of Milk Fat Synthesis

The regulatory mechanisms operating during lactation to control fatty acid synthesis are seemingly different than those operating during nonlactating states. This supposition is based on the reciprocal changes in lipogenesis that occur in adipose and mammary tissue during lactation; in other conditions, alterations in lipogenesis are generally similar in all effected tissues (179). No explanation to account for this apparent difference has been presented.

There are differences in the substrates used for milk fat synthesis depending on species, but most marked between ruminants and nonruminants (46). In the nonruminant lactating mammary gland, glucose is the major source of acetyl-CoA and NADPH for fatty acid synthesis. In the ruminant gland, acetate and 3-hydroxybutyrate are the principal precursors for fatty acid synthesis. Nevertheless, the general regulatory mechanisms that control the overall rate of fatty acid synthesis are believed similar in all species (47).

The critical role of acetyl-CoA carboxylase in modulating fatty acid synthesis in mammary tissue is well established in many species (33, 109). Its activity is believed to be rate-limiting for mammary lipogenesis. The action of this enzyme, generating

malonyl-CoA from acetyl-CoA, is the first step in fatty acid biosynthesis. A good correlation between total acetyl-CoA carboxylase and the rate of fatty acid synthesis has been demonstrated during pregnancy and lactation in several species (109, 110). Although the activity of acetyl-CoA carboxylase is known to be regulated by covalent modification of the enzyme (89), and there is evidence that the proportion of activated enzyme increases in mammary tissue during lactation (109), hormonal induction of fatty acid synthesis during lactation is primarily due to increased synthesis of this enzyme (106). Similarly, at weaning there is a marked decrease in the rate of fatty acid synthesis, but no change in the proportion of this enzyme in the active state (109). The synthesis of fatty acid synthetase also increases during lactation, as does the synthesis of most other major lipogenic enzymes (179).

The importance of prolactin and insulin in inducing changes in lipogenic enzyme biology has been demonstrated in many species (119, 179). Glucocorticoid also seems to play a stimulatory role in mammary lipogenesis, although this effect has not been consistently demonstrated *in vitro* (179). The mechanism by which this hormonal combination induces changes in the concentrations of lipogenic enzymes is unknown. However, it seems reasonable to expect that the changes are, at least in part, the result of hormone-mediated regulation of the expression of lipogenic enzyme genes, particularly the acetyl-CoA carboxylase gene or genes which encode the enzymes of the fatty acid synthetase complex. Although there is little information about hormonal induction of the enzymes responsible for fatty acid esterification into triacylglycerides in the mammary gland (71), it is likely that this induction also occurs during lactation.

Regulation of Lactose Synthesis

There has been a great deal of research effort directed at understanding the processes involved in lactose synthesis and their regulation (29, 98, 99). The lactose synthetase enzyme, which is composed of one molecule each of galactosyltransferase and α-lactalbumin, is responsible for lactose synthesis. The biosynthesis of lactose takes place in the Golgi apparatus and requires one molecule each of UDP-galactose and glucose. Thus, glucose is the essential precursor for lactose synthesis in all species.

The results of several studies with different species have led to the conclusion that neither galactosyltransferase nor α-lactalbumin are rate-limiting to lactose synthesis during lactation. Both enzymes, however, exhibit hormonal induction, initiated near the onset of lactation (97). The actions of prolactin and glucocorticoids, as well as removal of progesterone action, have been implicated as regulatory influences that initiate and maintain lactose synthesis during lactation (97, 98). The presence of apparent excess enzyme, however, suggests that the provision of substrates is the rate-limiting process in lactose synthesis.

Both *in vitro* and *in vivo* studies have addressed the question of whether glucose provision is in fact rate-limiting for lactose synthesis. Data from *in vitro* experiments indicate that the intracellular concentration of glucose does not saturate lactose synthetase (170, 186). Based on this finding, it has been suggested that the rate of glucose transport into the epithelial cell regulates the rate of lactose synthesis (99, 170, 186). However, the results of other *in vitro* work show that the epithelial cell glucose transporter is not saturated at the normal range of blood glucose concentration (98, 169). This finding supports a different mechanism, whereby changes in blood glucose concentration would affect changes in the rate of lactose synthesis.

Although both proposed mechanisms of lactose synthesis regulation seem plausible, neither is entirely supported by the results from *in vivo* studies. It has been demonstrated that mammary glucose uptake remains largely unchanged throughout most of lactation in sheep, despite the marked decline in lactose output toward the end of lactation (53). In addition, it has been shown that although milk yield drops by about 50% in fasted goats and cows, there is only a small decrease in blood glucose concentration (8, 69). Blood glucose concentration is also generally lower at peak lactation than it is later in lactation when lactose synthesis declines (53). Taken together, these observations suggest that the regulation of lactose synthesis must depend on the efficiency of the conversion of glucose into lactose. Although this is probably part of the overall regulatory mechanism, the synthesis of lactose is probably also regulated both directly by the rate of glucose transport and indirectly by blood

glucose concentration, as well as by feedback from metabolic systems of the cell. Regulation at multiple levels is most consistent with the general mechanism that provides glucose to other body tissues. It is also in accord with the observation that the rate of lactose synthesis generally parallels food intake patterns. Lactose synthesis also decreases during starvation and increases with refeeding (98). Future studies to characterize the regulation of the epithelial cell glucose transporter and its gene should lead to a better understanding of the regulation of lactose synthesis. Studies of the capillary endothelial cell glucose transporter in mammary vasculature may also prove fruitful in this regard (180).

GROWTH HORMONE BIOLOGY

It is well recognized that growth hormone (GH) produces multiple effects in target cells (79). It is also believed that the primary effects of GH are initiated through binding of GH to specific GH receptors on the surface of target cells (143). Post-receptor events, which supposedly transmit the GH signal, have not yet been identified. A GH serum-binding protein has been recently identified, but its function remains obscure (14, 72, 102).

In the past, the effects of GH have been generally classified as related to either growth or the metabolism of nutrients (79). However, it is now apparent that this classification system must be expanded to include other recently discovered GH actions. In particular, there is considerable evidence that GH plays a multifunctional role in several processes associated with reproduction (100). It is also well established that GH is involved in regulating differentiative processes in various cell types (48, 73, 100). This effect of GH is clearly growth-related in some instances, and in fact probably represents the primary effect of GH in the target cell growth process (63, 193). On the other hand, it is easy to envisage that the differentiative effect of GH in some target cells is unrelated to growth. The well established relationship between GH and insulin-like growth factor-I (IGF-I) reveals further diversity in GH action (37, 176).

The regulation of GH synthesis and secretion in the anterior pituitary is a complex process (24, 65, 118). Various hormones, growth factors, neuropeptides, and

neurotransmitter agents, as well as GH autoregulation are all involved in coordinating the synthesis and release of GH. For the most part, however, their function in the physiological secretion of the hormone is far from clear. It seems inevitable that the list of diverse GH effects will continue to grow, and in turn the complexity of regulation of GH function will become even more apparent.

LACTATION RESPONSE TO GROWTH HORMONE

In 1937 scientists first observed milk production increases in response to administering crude pituitary gland extracts to lactating cows (7). The constituent primarily responsible for this pituitary activity was identified as GH about 10 years later (192). There was hope that GH could be used to increase the milk supply in Great Britain during World War II; however, it was realized that even if all the GH from available bovine pituitary glands was administered to commercial dairy herds, it would have only increased the milk supply by .05% (192). Limited availability of GH also restricted its application in research. Nevertheless, the first experiment in which daily GH injections were administered for a long period (12 wk) to lactating cows was conducted in 1955 (27). In this study, milk yield of GH treated cows increased by 50% compared to control cows. Many subsequent experiments have also clearly demonstrated the ability of GH to increase milk production (12, 13, 41, 42, 129).

The renewed interest in this phenomenon over the last several years is primarily due to increased availability of GH. Advances in recombinant DNA technology resulted in the ability to produce limitless quantities of biologically active GH (149). So-called, recombinant GH is now available from commercial sources, including several major pharmaceutical companies. Both short- and long-term studies have demonstrated the efficacy of recombinant GH. Furthermore, the collective results of these studies suggest that GH treatment has no apparent adverse effects on animal health.

There is no scientific basis to expect that milk or milk products derived from GH-treated animals would adversely effect human health. This contention is most strongly supported by knowledge that all proteins, including GH from any source, are enzymatically cleaved into their constituent amino acids before absorption from the

digestive tract. Nevertheless, there has been consumer opposition to the notion of commercial use of GH in the dairy industry (52). The opposition to this GH application has primarily centered on questions concerning human safety. Such critical evaluation is of course warranted in all cases where new food technology presents a potential risk to human health. However, in view of scientific information that this GH application poses no risk to human health, it is somewhat discouraging to see consumer effort directed at opposing the issue. Clearly, any remaining health-related questions apply only to the GH-treated animal itself. Further consumer opposition should at least be redirected to focus on animal health.

It is important to recognize that misdirected or inappropriate opposition to this particular issue may ultimately impact negatively on the broader issue of biotechnology in agriculture. This emerging scientific field currently has general public support. Diminished public support would undoubtedly jeopardize continued research support from industry and government.

Since recombinant GH is one of the first products of biotechnology, it is imperative to educate the general public about its potential values to animal agriculture. Emphasis should be placed on its value strictly as a research tool. Most notably, it should be recognized that the purity of GH obtainable using biotechnology procedures is unprecedented. This degree of purity of GH allows, for the first time, definitive studies on its role in processes of animal physiology. Knowledge of these processes may ultimately lead to the development of novel approaches to increase the efficiency of milk production. Such progress may in fact be one of the only ways to obviate the use of other approaches, such as GH injection. Clearly this is a prime example of how creating a better public awareness of a less obvious, but potentially important value of biotechnology could lead to further support, and in turn benefit agricultural biotechnology in general.

The issue of GH purity is relevant to a survey of the studies which have examined the lactation response to GH injection. Specifically, it questions the biological significance of some of the effects of GH observed in experiments using pituitary-

derived GH preparations. Not surprisingly, there are apparent discrepancies regarding several of the physiological responses supposedly induced by GH. The most discrepant results are those concerning either the presence or absence of acute GH effects. When observed, such acute effects were usually transitory changes in blood concentrations of free fatty acids, glucose, or insulin (20, 22, 131, 134). These effects have been commonly referred to as the diabetogenic and lipolytic activities of GH. This issue has only recently been resolved by the results of studies using recombinant GH. It is established that the acute effects are not properties of GH, but are due to artifactual modification of the GH molecule.

A general pattern of physiological effects has emerged from long-term studies in which lactating cows were treated with daily GH injections. During long-term treatment, feed intake gradually increases to support the higher milk production (11, 12, 129). The change in feed intake results in no differences in live weight changes between treated and control animals over the treatment period. There are no apparent differences in the efficiency of digestion, maintenance requirements of the animals, or the efficiency of milk synthesis (13). Overall, the gross feed efficiency of treated animals is increased because maintenance represents a smaller proportion of the consumed nutrients.

During GH treatment, mammary blood flow and cardiac output increase, as does the proportion of cardiac output diverted to the mammary gland (40, 68, 114). Although it has been suggested that GH induces these circulatory changes (68, 114), thereby providing an increased supply of rate-limiting substrates for milk synthesis, this seems unlikely. It is generally accepted that organ blood flow is primarily regulated by the oxygen requirements and carbon dioxide production of the organ. Thus, the observed circulatory changes probably reflect the demands of increased milk synthesis. Furthermore, with the exception of periods of extreme nutrient deprivation, blood concentrations of substrates for milk synthesis are not rate-limiting (129). Even postruminal infusion of nutrients during GH treatment does not increase production above that with GH alone (105, 130).

During GH treatment, there are generally no significant changes in blood concentrations of insulin, prolactin, glucagon, thyroxine, triiodothyronine, and glucocorticoids (13). There are also generally no changes in blood concentrations of nutrients such as glucose, 3-hydroxybutyrate, lactate, and urea (13). However, if cows are in a negative energy balance when GH treatment begins, or if GH treatment results in negative energy balance, then blood concentration of free fatty acids is chronically increased (129).

The concentrations of milk protein, lactose, and fat generally increase parallel to the increase in milk yield (51). In negative energy balance, however, the milk fat increase is greater, and the milk protein increase is less than the corresponding milk yield increase (51). Furthermore, when cows are in a negative energy balance there is an increase in the proportion of long chain fatty acids in milk triacylglycerides (22). This change in the composition of milk fat is presumably the result of lipid mobilized from adipose tissue being used to synthesize mammary triacylglycerides. Both the characteristic long chain fatty acid composition of triacylglycerides in adipose tissue (13), and the chronic increase in free fatty acid blood concentration during negative energy balance support this contention. Although blood concentrations of calcium and phosphorous are unchanged during treatment with GH, their concentrations in milk increase in proportion to that of milk yield (129). Similar increases are also observed for other nutritionally important minerals (129).

During treatment with GH there is additional demand for precursors to support the increased synthesis of milk constituents. The actual sources of these precursors have not been clearly established. However, changes in the irreversible loss and oxidation rates of glucose and free fatty acids can quantitatively account for the increases in at least lactose and milk fat (19, 129). Gluconeogenesis from amino acids and propionate may provide some of the additional glucose for lactose synthesis. Glycerol produced from the hydrolysis of adipose tissue triacylglycerides could also provide another source of glucose.

It has not been determined if amino acids are spared from oxidation during GH treatment. This could provide the additional source of amino acids for milk protein synthesis. Alternatively, these amino acids may be derived from body protein reserves. This is the most likely source when cows are in a negative nitrogen balance, as well as early in a GH treatment period when feed intake has not yet increased. As feed intake increases, however, the additional precursors for increased milk synthesis are likely provided mostly by consumed nutrients.

During lactation, changes in metabolism take place to provide precursors for milk synthesis as well as substrates for use in energy-requiring mammary cell processes (16, 132). It is easy to envisage that the same metabolic adaptations are simply enhanced during GH treatment, to provide additional precursors and substrates to support increased milk synthesis. Body weight decreases early in normal lactation to support milk synthesis. However, later in lactation when increased feed intake is sufficient to support milk synthesis, body weight begins to increase. GH treatment and increased milk synthesis are also initially accompanied by a decrease in body weight (11). As in normal lactation, feed intake gradually increases during GH treatment and eventually becomes sufficient to support increased milk synthesis. Thus, the physiological adaptations which occur during GH treatment seem to be the same adaptations which occur during early lactation without GH treatment.

It is also possible that the physiological events induced by GH are entirely different than those occurring during early lactation, although this seems unlikely. Assuming the underlying physiological processes are the same, however, it follows that their regulation would be mechanistically similar. In accord with this idea, there is an increase in the responsiveness of adipose tissue to epinephrine during early lactation (81, 116, 178), and even a further increase in responsiveness as a result of GH treatment (107). In both instances, the increased responsiveness presumably reflects an adaptation in adipose tissue required to supply additional lipids for increased oxidation and milk fat synthesis. Increased oxidation of lipids from adipose tissue would spare glucose and amino acids for increased lactose and milk protein synthesis.

The similarities in metabolic adaptation between early lactation and GH-treated cows point to a common mechanism for regulating nutrient supply to support increases in milk synthesis. Indeed, there is an increase in GH concentration in blood during early lactation, and the increase is highly correlated with milk yield (19, 67, 78, 144, 146). The nature of the role of GH in this common mechanism, which alters metabolic processes to support increases in milk synthesis, remains unknown.

MECHANISM OF GROWTH HORMONE ACTION

Simply providing the mammary gland with additional nutrients does not necessarily stimulate milk production. It is this fundamental concept that holds the key to understanding the mechanism by which GH stimulates increased milk production. A prerequisite for increased milk production is either an increase in the number of functionally differentiated alveolar epithelial cells or in the overall biosynthetic activity of existing cells, or a combination of both. The essential nature of these changes are readily apparent, especially considering the magnitude of the milk yield increase induced by GH treatment.

The inevitable question concerning how GH actually stimulates milk yield is whether changes in the mammary gland direct metabolic changes in the body, or whether changes in the body direct changes in the mammary gland. Lack of knowledge about the intricacies of higher level control of lactation makes it difficult to offer adequate explanations for either answer to this question. Nevertheless, explanations are frequently presented (13, 129). Of those presented, the most common explanation is that GH induces changes in both body tissues and mammary tissue. Furthermore, depending on the change, the induction is either direct or indirect. This common general explanation seems to cover most, if not all possibilities and therefore avoids critical evaluation.

An attractive, alternative hypothesis includes the concept of signals from the mammary gland being ultimately responsible for directing the major changes in other body tissues. This hypothesis offers a conceptual basis for understanding the mechanism of GH action in lactating cows treated with GH. Specifically, it proposes

that either hormonal- or metabolic-related signals originate from mammary tissue and initiate mechanisms that coordinate metabolic adaptations in supporting tissues. The rationale for this hypothesis is illustrated by analogy to the situation in a monotocous species carrying twin fetuses. The increase in supply of blood and nutrients to support the two fetuses is regulated by maternal mechanisms, whereas the overall process is initiated simply by the presence of more fetal tissue. Thus, unknown signals must originate from fetal tissue and then initiate the maternal mechanisms that coordinate the appropriate physiological adaptations. It is tempting to speculate that the original signals from fetal, as well as mammary tissue are simply related to increased oxygen demand or carbon dioxide output; however, considering the unique nature of both these tissues and the lack of understanding of the mechanisms regulating their growth and function, this speculation is largely without theoretical basis.

Part of the proposed hypothesis is the concept that the first major event following GH injection is action on the mammary gland. Both a direct GH action on mammary tissue and a secondary action involving one or more intermediates are consistent with this concept. A prerequisite to direct GH action would be the presence of specific GH receptors on mammary cells. Also, because the most likely candidates for mediator roles are other hormones, their action would require the presence of corresponding receptors. Whether the response involves direct GH action or the action of a mediator, it would be realized as either an increase in the number of active cells or in the overall biosynthetic activity of the gland.

The results of several experiments, in which mammary membranes were solubilized to evaluate their ability to selectively bind bovine GH, have led to the conclusion that specific GH receptors are not present in the bovine mammary gland (3, 59, 86, 88). This conclusion seems premature, especially considering the heterogeneous population of cell types in mammary tissue, some of which have been shown to posses GH receptors in other tissues (143). In addition, GH receptor binding studies are generally difficult to perform (143). The use of heterogeneous binding systems to study GH receptor binding is also controversial.

Another concept of the proposed hypothesis is that GH plays a role in mammary growth or function, either directly or indirectly through the actions of an intermediate. The question of whether an action of GH influences mammary processes has been examined in both *in vitro* and *in vivo* experiments (3, 9, 60, 61, 108, 158, 173). Collectively, the results of experiments involving several different *in vivo* approaches have clearly established a role for GH in both mammary growth and function. The different *in vivo* approaches have involved replacement therapy in endocrine ablated animals, mammary tissue or cell transplantation into athymic mice, and GH treatment of endocrine intact animals at different stages of mammary development and lactation. Although various GH effects have been demonstrated through the use of each of these approaches, none of them can be used to demonstrate a direct GH action. Even the athymic mouse system with tissue and hormones from other species may involve secondary actions by endogenous intermediates.

The results of *in vitro* experiments with rat, mouse, and rabbit mammary tissue suggest that GH has a direct effect on mammary tissue (120, 173). On the other hand, results from *in vitro* studies using bovine mammary tissue are inconsistent. Nevertheless, the results are usually interpreted to suggest that GH has no direct effects on bovine mammary tissue (3, 60, 61). This interpretation does not account for the *in vitro* effects that have been reported. GH has been shown to affect casein and milk fat synthesis, and lactose secretion in bovine mammary explants, as well as casein synthesis in goat mammary explants (60, 61, 158). The inconsistent interpretation of GH effects on mammary tissue *in vitro* is due, at least in part, to suspicion of contamination in pituitary-derived GH preparations (9, 61). The possibility that cultured mammary tissue differs in certain ways from the tissue *in vivo* is also an important consideration. The use of recombinant GH and further improvements in mammary tissue and cell culture techniques should resolve these remaining uncertainties.

The fundamental knowledge that marked changes occur in mammary tissue as a result of GH treatment provides a strong impetus for researchers to determine the exact

nature of these changes. Unfortunately, this has not yet been attempted. The application of techniques such as computerized x-ray tomography and magnetic resonance imaging would likely provide insight into the nature of the mammary changes.

Identification of the signaling molecules involved in inducing the mammary changes is also of great interest. However, the lack of supporting evidence for a direct GH action in inducing the changes raises interest in the possibility of mediator involvement. The most obvious candidate for this role is insulin-like growth factor-I (IGF-I). Support for this idea is provided by the recent finding that the effect of GH on tibial cartilage growth in the epiphyseal growth plate of hypophysectomized rats is the result of GH-induced local IGF-I production (146). That GH may exert a similar indirect action on other target tissues is suggested by findings that IGF-I has mitogenic action on several types of cultured cells, which also produce IGF-I in response to GH (1, 34). Furthermore, it has also been demonstrated that several different rat tissues produce IGF-I in response to GH (36). Collectively, these observations provide a basis to speculate that IGF-I may also mediate the action of GH on bovine mammary tissue.

The observation that four different human breast cell lines have specific IGF receptors (type I) provided the first convincing evidence to suggest that IGF-I may play a role in regulating mammary processes (57). Moreover, this study also showed that IGF-I stimulated an increase in DNA synthesis in all four cell lines. It has also been demonstrated that IGF-I promotes the accumulation of mRNA in the rough endoplasmic reticulum of mouse mammary explants (25). In addition, the combination of IGF-I, prolactin, and cortisol has been shown to stimulate an increase in the rate of carrier-mediated glucose transport in cultured alveolar epithelial cells isolated from pregnant mice (135).

Although there is considerable evidence to propose an IGF-I role in human and mouse mammary gland biology, there is no direct evidence to support such a role in the bovine gland. However, there is a basis to speculate that cows treated with GH would at least have an increased blood IGF-I concentration. Several species, including

humans, sheep, mice, and rats exhibit a marked serum IGF-I induction response to GH injection (37). It is therefore conceivable that dairy cows may also exhibit a similar response.

Our current state of knowledge about the role of GH in mammary biology is very far from complete. However, the observation that GH injection alone alters mammary processes so markedly during lactation indicates clearly that the action of GH plays an integral role in mammary biology. Accordingly, the overall objective of the research of this thesis is to gain a better understanding of this specific role of GH. To achieve this objective a research strategy based on inference from observed data was developed. The strategy involves the analysis of several specific suppositions. Data collected from these analyses will allow a critical evaluation of the proposed hypothesis. It is hoped that the findings of this evaluation will contribute to a better understanding of the mechanism of GH action during lactation. Such knowledge may in turn provide insight into the role of GH in other stages of mammary development, and thus mammary biology in general.

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Effect of Bovine Growth Hormone on the Distribution of Immunoreactive Insulin-Like Growth Factor-I in Lactating Bovine Mammary Tissue*

INTRODUCTION

Much recent attention has been paid to the ability of injected bovine growth hormone (bGH) to stimulate milk production markedly in lactating dairy cattle (4). This phenomenon has considerable biological interest as well as economic significance for the dairy industry. However, the biochemical details of bGH action in the mammary gland are not well understood. Enhanced substrate processing by the mammary epithelial cells or increased numbers of epithelial cells are necessary for milk production to increase. However, bGH cannot interact directly with the epithelial cells, because they apparently do not possess specific bGH receptors (2, 15, 22). To stimulate milk production, bGH must therefore act directly on other mammary cell types or non-mammary cells to influence indirectly the growth and activity of mammary epithelial cells.

A number of recent studies indicate that the stimulatory effect of GH on tibial cartilage growth in the epiphyseal growth plate of hypophysectomized rats is the result of local GH-induced insulin-like growth factor-I (IGF-I) production (20, 26). That GH may exert a similar indirect action on other target tissues is suggested by the observations that IGF-I has mitogenic action on several types of cultured cells, such as human fibroblasts and porcine smooth muscle cells, that also produce IGF-I in response to GH (1, 7). Furthermore, multiple rat tissues (e.g., kidney, lung, heart, liver, testes) produce IGF-I in response to GH (12). The abundance of IGF-I mRNA in a variety of rat tissues has also been shown to be regulated by GH (19). Thus, it is possible that GH acts indirectly on a number of tissues by stimulating local production of IGF-I.

The presence of specific IGF-I receptors on bovine mammary epithelial cells (6) and the recent finding that IGF-I exerts mitogenic action on these cells in culture (5)

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point to a role for IGF-I in the regulation of mammary epithelial cell growth in dairy cattle. In addition, bGH administration to lactating dairy cattle results in an increase in serum IGF-I concentration (11). The objectives of this study were to characterize the cell-specific localization of immunoreactive IGF-I in mammary tissue of lactating dairy cattle and to determine the effect of bGH injections on the distribution of IGF-I in mammary tissue.

MATERIALS AND METHODS

Animals and Experimental Design

Experiment 1. An experiment was undertaken to determine the time course response in serum IGF-I concentration to a single bGH injection. Four lactating Holstein cows (second lactation) between 40 and 53 d postpartum were used. Animals were fed a complete mixed diet containing 60% concentrate and 40% forage. All animals received a single saline injection (control) followed by periodic blood sampling for 24 h, and then a single bGH injection followed by periodic blood sampling for 72 h. The bGH used in all experiments was a preparation produced by recombinant DNA technology (Lot No. PR6776C-169A, American Cyanamid Company, Princeton, NJ). Physiological saline (2.0 ml, .9% NaCl) and bGH (20.6 mg in 2.0 ml physiological saline) were administered as single subcutaneous injections in the shoulder area. The bGH was dissolved in physiological saline just prior to administration.

A catheter for blood withdrawal was inserted into the jugular vein of each animal the day before the experiment started. Blood samples (10 ml) were taken just prior to administering the saline injection and at 4 h intervals following injection. After bGH injection, blood samples were taken at 2 or 4 h intervals. Blood samples were allowed to clot at room temperature for 4 h and centrifuged at 1000 × *g* for 15 min. Serum was stored at -20°C until processed for use in the IGF-I radioimmunoassay (RIA).

Experiment 2. Three Holstein cows in second or later lactation were used. Two animals were in late lactation (240 and 276 d postpartum) and one was in early lactation (97 d postpartum). Animals were fed a complete mixed diet containing 50%

concentrate and 50% forage. All animals received daily saline injections for 3 consecutive d (control) and then bGH (20.6 mg/d) injections for another 3 consecutive d. Injection volumes and administration route were the same as in Experiment 1. Mammary tissue was sampled by percutaneous biopsy 10 h after both the last saline and bGH injections. Animals were milked 3 to 4 h prior to taking mammary biopsies. Blood samples (10 ml) were withdrawn by puncture of the coccygeal vein or artery 4 h before the first saline injection and at 12 h intervals over the entire experimental period. Blood samples were collected into siliconized Vacutainers (Beckton Dickinson and Company, Rutherford, NJ) and serum obtained and stored as in Experiment 1.

Analysis of Serum IGF-I Concentration

The IGF-I in serum samples was separated from its serum binding proteins by acid gel filtration using a previously published procedure (30) and suggested modifications in the procedure (9). The IGF-I was measured by RIA using antiserum (UB 286) provided by J.J. Van Wyk and L.E. Underwood, University of North Carolina, and distributed by the National Hormone and Pituitary Program of the National Institutes of Arthritis, Diabetes, Digestive, and Kidney Diseases. This antiserum is highly specific for IGF-I and has only .5% crossreactivity with IGF-II.

The RIA for IGF-I was carried out in 12 × 75-mm polystyrene tubes (Fisher Scientific, Edmonton, AB) using a nonequilibrium technique (14). The assay buffer contained .05% Tween 20 (Fisher Scientific) as a substitute for bovine serum albumin (9). An appropriate volume of the gel filtrate of serum was dried under nitrogen gas in an assay tube and resuspended in 100 µl of assay buffer to obtain 1:200 dilution. Standards (.125 to 5.0 ng/ml IGF-I in 100 µl assay buffer) or unknowns were preincubated with the antiserum (1:8000 final dilution) for 3 d at 4°C before addition of [^{125}I]IGF-I. The standard used was a preparation of IGF-I synthesized by solid phase methodology and purified by high performance liquid chromatography (Lot No. 588C, Bachem Fine Chemicals, Torrance, CA). This preparation was also used for iodination (^{125}I, ICN Radiochemicals, Irvince, CA) by the chloramine-T method (30). Approximately

10,000 cpm [^{125}I]IGF-I in 100 µl of assay buffer were added and the incubation carried on for another 24 h at 4°C. The final volume of the reaction mixture was .5 ml.

Separation of antibody-bound and free [^{125}I]IGF-I was accomplished by adding 100 µl goat antirabbit gamma globulin (diluted 1:25 in assay buffer, Gibco Canada Inc., Burlington, ON) and 100 µl of normal rabbit serum (diluted 1:150 in assay buffer). After 24 h at 4°C the tubes were centrifuged (3000 × *g*, 15 min, 4°C), the supernatant aspirated, and the precipitate counted in a gamma counter. The gel filtrate of each serum sample was tested in duplicate. Displacement of [^{125}I]IGF-I by gel filtrates of bovine serum was parallel to IGF-I standards. All samples were assayed in a single assay. The within assay coefficient of variation was 3.5%. Recovery of synthetic IGF-I added to bovine serum, incubated for 1 h at room temperature, and chromatographed was 97%. Recovery of [^{125}I]IGF-I from bovine serum was 93% of the added counts using this method. The reported concentration values are not corrected for the apparent loss.

Mammary Biopsy Technique

Parenchymal tissue was aseptically excised from the left rear quarter of the mammary gland of each animal using a Tru-Cut biopsy needle (Travenol Laboratories, Deerfield, IL). The second biopsy was taken approximately 10 cm distant (to the right) from the first biopsy site. Tissue samples (approximately 15 mm long × 1 mm o.d.) were taken from an area about 15 cm dorsal to the base of the teat at a depth of approximately 8 cm. Sampling from this location yields tissue relatively free of large ducts (22). Prior to each tissue excision, animals received a local anesthetic (2.0 ml of 2% lidocaine) that was injected under the skin surrounding the site of incision and biopsy needle insertion. An incision (approximately .5 cm) was required to facilitate biopsy needle insertion. Tissue excision caused no external bleeding. In a preliminary experiment, postmortem mammary tissue was examined the day after performing a single percutaneous biopsy. Evidence of mild tissue trauma (i.e., slight reddening) was confined to the immediate area around the excision site (maximum 1 cm radius). Puncture track tissue damage was not visible.

Morphological Techniques

Tissue Fixation and Cryoprotection. Excised tissue was immediately placed in a fixative solution of 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.2, and immersion-fixed for 90 min at 4°C. The fixative solution was prepared fresh daily using powdered paraformaldehyde (Fisher Scientific Company). Following fixation, tissue was washed with three changes of phosphate buffered saline (PBS, pH 7.4), 30 min each, at 4°C. Tissue was then cryoprotected by immersion in 30% sucrose (wt/vol) in PBS for 3 h at 4°C.

Embedding and Sectioning. Tissue was mounted in OTC compound (Ames Company, Elkhart, IN) by rapidly freezing in isopentane (-150°C) cooled by liquid nitrogen. Mounted tissue was stored in air tight polypropylene bags at -70°C until cryosectioning. Tissue sections for immunocytochemistry were cut at 8 µm in a cryostat (Ames Company) at -20°C. Serial sections were periodically taken and stained with hematoxylin and eosin for examination of tissue morphology and preservation by light microscopy.

Immunocytochemistry. After cryosectioning, frozen tissue sections were picked up onto rubber cement (Lepage's Limited, Bramalea, ON) coated coverslips and allowed to dry for 30 min at room temperature. Immediately after drying, sections were washed in PBS for 30 min at room temperature. To minimize nonspecific binding of the secondary antibody, sections were incubated with normal goat serum (30% in PBS) for 30 min at room temperature. Sections were then incubated with primary antibody (K1792, KabiVitrum, Stockholm, Sweden) at 1:1000 dilution in PBS for 18 h in a moistened chamber at 4°C. The K1792 antiserum was produced by immunizing rabbits with a synthetic peptide corresponding to the carboxyterminal amino acids 57 to 70 of human IGF-I; the amino acid sequence of bovine IGF-I has recently been shown to be identical to that of human IGF-I (18).

After incubation with primary antibody, sections were washed three times with PBS, 15 min each at room temperature. Secondary antibody (fluorescein isothyocyanate (FITC)-labeled goat antibody to rabbit immunoglobulin G, Sigma Chemical Company, St.

Louis, MO) at 1:30 dilution in PBS was incubated on sections for 60 min at room temperature. The incubation with secondary antibody, as well as all subsequent procedures in the immunocytochemical protocol, was carried out in the dark. After incubation with the secondary antibody, sections were washed three times with PBS, 15 min each at room temperature. A final wash comprising three brief immersions in distilled water was performed before mounting sections. Sections were mounted in glycerol-containing paraphenylenediamine to reduce immunofluorescent fading (21).

Immunocytochemical Controls. Controls to check the specificity of the immunocytochemical reactions included: omission of the primary or secondary antibodies, substitution of the primary antibody with serum from an unimmunized rabbit, and incubation with antiserum (K1792) depleted of anti-IGF-I antibodies by solid phase immunoabsorption (29). The solid phase immunoabsorbent was prepared by coupling 20 μg of synthetic IGF-I in PBS (Bachem Fine Chemicals) to cyanogen bromide-activated Sepharose 4B (Sigma Chemical Company) using a previously described protocol (29). A control Sepharose gel was prepared by the same protocol, using PBS containing no IGF-I. Liquid phase absorption was also performed by incubating K1792 antiserum (1:1000) with .1, .25, .5, 1.0, 1.5, 2.0, and 5.0 μg/ml IGF-I for either 30 min or 3 h at 37°C prior to applying antiserum to the tissue sections. Liquid phase absorption was also performed by incubating K1792 antiserum with IGF-I for 30 min at 37°C and then for 18 h at 4°C. Following the 18 h incubation, the antiserum containing IGF-I was centrifuged at 8000 × *g* for 5 min (4°C), and the supernatant was removed and applied to tissue sections.

The antiserum used has been previously well characterized and shown to be highly specific for IGF-I (17). Specificity of the antiserum has been demonstrated by RIA, double-immunodiffusion, Western and dot blotting (17). Relaxin, IGF-II, and proinsulin were shown to have no significant crossreactivity with the antiserum.

Fluorescence Microscopy and Photography. Fluorescent microscopic images were recorded on Kodak Ektachrome 200 film using a Leitz Dialux photomicroscope equipped with epi-illumination for fluorescence microscopy. A standard exciter/barrier filter

combination for fluorescein was used. All fluorescent photomicrography was performed immediately after sections were mounted. Preparations were observed with 25X and 40X PL, and 100X NPL oil immersion objectives. Typical exposures were about 10 s. Images of control sections were recorded using the same exposure times.

RESULTS

Effect of Bovine Growth Hormone on Concentration of Insulin-Like Growth Factor-I in Serum

The effect of a single bGH injection on serum IGF-I concentration is shown in Figure II-1. The basal concentration of IGF-I averaged throughout the period before bGH injection was 261.7 ± 12.3 (SEM) ng/ml; an increase was first detected 8 h after the bGH injection and the maximum value (509.0 ± 26.4 ng/ml) was reached at 18 h. The IGF-I concentration at 72 h after injection (333.8 ± 33.8 ng/ml) was still slightly higher than the basal value.

One of the cows (late lactation) in Experiment 2 failed to show a significant rise of serum IGF-I above the basal value after any of the bGH injections (data not shown). This cow exhibited nervousness during blood sampling, injection administration, and the biopsy procedure. At initiation of bGH treatment, this cow's feed intake had decreased to less than half of the intake at the start of the experiment. Because fasting and protein-calorie deficiency are known to inhibit markedly the GH-stimulated rise of serum IGF-I (25), lack of an IGF-I response in this animal was assumed to be related to her nutrient-deprived state.

The effect of three consecutive daily bGH injections on serum IGF-I concentration is shown in Figure II-2. The basal concentration of IGF-I averaged throughout the control period (saline treatment) was 372.9 ± 12.2 ng/ml. By 8 h after the first bGH injection, an increase in the serum IGF-I concentration was detected. A further elevation in IGF-I concentration was observed 20 h after the first injection and again within 8 h after the second bGH injection. The magnitude of the response to the second injection was similar to that observed for the initial injection. An additional injection of bGH did not seemingly result in a further increase in the serum IGF-I

concentration; however, the peak response was maintained and still present at 8 h after the last bGH injection. At peak response, serum IGF-I concentration was raised about twofold above the basal value. Based on the information obtained concerning the time course of the IGF-I response to bGH, serum IGF-I concentrations were at or near peak response values when the second mammary biopsies were taken.

Morphology of Lactating Mammary Tissue

Light microscopic examination of cryostat sections of mammary biopsies stained with hematoxylin and eosin revealed satisfactory morphological preservation (Figure II-3). Tissue was characterized by distended alveoli that were lined by a single layer of epithelial cells. Varying amounts of stainable secretory material and some cell debris were observed within the lumens of alveoli. Epithelial cells were frequently seen to contain large supranuclear fat globules abutting into the alveolar lumen. Myoepithelial cells, that lie between the bases of the epithelial cells, were difficult to identify because in the distended alveoli they are mostly flattened beyond recognition. When observed, however, they were seen to contain small nuclei surrounded by clear cytoplasm.

Alveoli were surrounded by compressed connective tissue of the intralobular stroma. Numerous small blood vessels and dilated capillaries were present in the intralobular stroma. Fibroblast-like cells as well as other cell types, presumably lymphocytes, blast cells, and plasmacytes, were also observed in the stromal matrix.

Distribution of Immunoreactive IGF-I in Control Tissue

In mammary tissue from saline-injected animals the distribution of IGF-I immunoreactive material was qualitatively similar for all animals. The IGF-I immunoreactivity was primarily seen associated with stromal elements (Figure II-4). The most intense staining was consistently present as a cytoplasmic accumulation of aggregate granules in cells located in the intralobular stroma. Some intralobular cells also showed circumferential staining patterns. This immunofluorescent pattern may represent IGF-I binding to cell surface receptors. In contrast, the attenuated fibroblasts that lie adjacent to the connective tissue surrounding each individual alveolus, were seemingly negative.

Small blood vessels showed moderate to high IGF-I immunoreactivity. Staining of the media in vessel walls may represent IGF-I associated with smooth muscle cells or extracellular IGF-I in the connective tissue or both. Occasionally IGF-I immunoreactive material was seen in the tunica intima, presumably associated with the endothelial cells. Capillaries in the intralobular stroma also stained. This staining presumably represents IGF-I associated with endothelial cells of capillaries, or with the modified smooth muscle cells (i.e., pericytes) that surround capillaries.

Myoepithelial cells, which surround the glandular alveolus, were seemingly negative. Occasional fine elastic fibers located in the intralobular stroma showed slight autofluorescence (yellowish color) in control sections. Such autofluorescence was very rarely detected in sections stained with antiserum because of faint staining of other elements in the intralobular stroma. It is possible that some intralobular stromal staining represents IGF-I immunoreactivity associated with plasma cells (e.g., lymphocytes), fibroblastic processes, or simply free IGF-I in this extracellular matrix.

Although immunoreactive IGF-I was not detected in the cytoplasm of most epithelial cells, some displayed a few discrete cytoplasmic fluorescent granules (Figure II-4B). In contrast, the circumferential distribution of intense IGF-I immunoreactivity around each individual alveolus suggests that this growth factor is mainly associated with the basal plasma membrane of epithelial cells. Alternatively, this fluorescent pattern may represent IGF-I immunoreactive material in periductular connective tissue.

Distribution of Immunoreactive Insulin-Like Growth Factor-I in Bovine Growth Hormone-Treated Tissue

The distribution of IGF-I detected by immunofluorescence in tissue obtained after bGH treatment was qualitatively similar for all animals. The distribution was also similar in many respects to that seen in control tissue. Cells and tissue elements in the intralobular stroma that were IGF-I immunoreactive in control tissue were also stained in tissue obtained after bGH treatment (Figure II-5). The epithelial cells, however, more frequently displayed cytoplasmic IGF-I immunofluorescence when compared with epithelial cells in control tissue (compare Figure II-4 and 5). Most

epithelial cells contained a diffuse distribution of IGF-I immunofluorescent granules. Sparing of nuclei and probable lipid globules or secretory vesicles could be visualized in these cells. Although convolutions in the basal plasma membrane were not visible at the resolution used, the rounded bases of bulging epithelial cells were occasionally discernable, presumably because of immunofluorescent IFG-I molecules bound to basal plasma membrane receptor sites. Occasionally, cells occupying locations typical of myoepithelial cells in lactating mammary tissue were observed to contain a moderate amount of diffuse IGF-I-stainable material. Although the method used here is not strictly quantitative, there also appeared to be an increase in overall staining intensity after bGH treatment (compare Figure II-4 and 5).

Immunocytochemical Controls

Control sections showed no IGF-I immunoreactivity when the primary antibody K1792 or the secondary antibody was omitted (Figures 6A, B). Incubation with serum from an unimmunized rabbit resulted in only a low level of nonspecific background staining (Figure II-6C). Liquid phase absorption of K1792 with various concentrations of IGF-I and incubation conditions failed to abolish staining (not shown). Staining was sometimes enhanced by this treatment. After solid phase absorption of K1792, specific staining was abolished, and only background staining was observed (Figure II-6D). Preincubation of K1792 with the control sepharose gel did not change either the intensity or pattern of staining (not shown). The failure of liquid phase absorption to abolish staining and, in some instances the increase of staining after liquid phase absorption have been reported by other researchers using antibodies against different antigens (27, 28). The added antigen used for liquid phase absorption likely explains enhanced staining. Failure of liquid phase absorption to abolish staining is thought to be caused by the presence of high antigen density in the tissue section, resulting in higher functional affinity of specific antibodies for antigen in the tissue than for antigen in the solution (27).

DISCUSSION

Indirect immunofluorescence was used to determine the localization pattern of immunoreactive IGF-I in mammary tissue obtained from both normal and bGH-treated lactating dairy cattle. The immunocytochemical results show that under normal conditions IGF-I immunoreactive material is present in lactating bovine mammary tissue, and that it is primarily found associated with stromal elements in this tissue. Injection of cows with bGH altered the distribution of IGF-I immunoreactivity in this tissue. After bGH treatment, IGF-I immunoreactivity was still detected in mammary stroma; however, there was also prominent staining in the alveolar epithelium. Most IGF-I immunoreactivity in the alveolar epithelium was present as diffuse cytoplasmic granules in epithelial cells, but occasionally myoepithelial cells also displayed immuno-stained material. The observed changes in the distribution of IGF-I in mammary tissue correspond temporally with increased circulating IGF-I and milk production responses, which are consistently observed by three d after starting daily bGH injections at dosages similar to that used in the present study (4).

The subcellular localization of IGF-I immunoreactivity present in the cytoplasm of epithelial cells after bGH treatment could not be determined with the method used in the present study. Regardless of its subcellular location, this staining pattern suggests either local synthesis or internalization of IGF-I stainable material. Local synthesis cannot be excluded, but bGH-induced local synthesis in the epithelial cells is considered to be unlikely because these cells apparently do not possess specific bGH receptors (2, 15, 22). Internalization of receptor bound IGF-I is more likely because these cells possess specific IGF-I receptors (6). Furthermore, because biological action of IGF-I on target cells presumably requires liqand-receptor binding and internalization, the finding that IGF-I has mitogenic action on cultured bovine mammary epithelial cells also suggests that the staining observed in these cells is the result of internalization of receptor bound IGF-I (5).

The source of the IGF-I that was observed in mammary epithelial cells after bGH treatment is unknown. The twofold increase in serum IGF-I concentration in bGH

treated animals suggests that the IGF-I found in epithelial cells was synthesized in other tissues and delivered to the mammary gland by the blood. But it is also possible that this IGF-I is both bloodborne and locally synthesized IGF-I that has been internalized by the epithelial cells. Consistent with the hypothesis of local IGF-I synthesis is our recent finding that IGF-I mRNA is synthesized in lactating bovine mammary tissue (unpublished observation). Regardless of the source, the presence of more IGF-I in mammary epithelial cells after bGH treatment seems to have considerable relevance to the biological action of bGH on mammary tissue. The results of this study suggest that IGF-I mediates the stimulatory effect of bGH on mammary tissue. Such a mechanism would likely involve bGH-induced IGF-I interacting directly with the epithelial cells to stimulate mitogenesis, or alter specific metabolic processes.

Some of the IGF-I immunnoreactivity detected in mammary stroma in the present study may represent IGF-I associated with intralobular fibroblasts. Consistent with this possibility is evidence that fibroblasts in other species have specific IGF-I receptors (24) and that cultured fibroblasts synthesize IGF-I (1). Insulin-like growth factor-I has a wide range of biological effects on fibroblasts in culture (8). Thus, these observations not only suggest that mammary fibroblasts would stain for IGF-I, but may also explain both the circumferential staining pattern and cytoplasmic staining seen in unidentified stromal cells in this study. That fibroblasts in culture synthesize IGF-I in response to GH further suggests that these cells in mammary tissue may respond to bGH in a similar fashion. Currently, we are undertaking *in situ* hybridization studies to examine which cell types synthesize IGF-I mRNA in bovine mammary tissue utilizing a cloned IGF-I gene probe.

Both before and after bGH treatment, IGF-I immunoreactivity was also visualized in small blood vessels and capillaries located in the intralobular and interlobular mammary stroma. This observation agrees with the recent finding that several elements in rat blood vessel walls display immunofluorescence when stained by indirect immunofluorescence with specific anti-IGF-I antibodies (16). Mammary vascular staining after bGH injection, a treatment known to increase mammary blood flow in

lactating dairy cattle (10), suggests a role for IGF-I in adaptation to increased blood supply. This idea is also supported by the finding that IGF-I immunoreactivity is increased in rat blood vessels in response to a vascular load (16). An IGF-I role in other mammary vascular processes is suggested by the observations that IGF-I has mitogenic action, and alters various metabolic processes in cultured capillary endothelial cells isolated from bovine retinas (23) and adipose tissue (3). These findings and the results of the present study suggest that IGF-I may be involved in regulating various vascular processes during both normal and bGH-stimulated lactation in dairy cattle.

An alternative hypothesis to explain the vascular staining, both before and after bGH treatment, is that it represents bloodborne IGF-I being stored or processed by the endothelial cells. This is supported by the recent finding that cultured endothelial cells from bovine capillaries and blood vessels have specific surface receptors for IGF-I as well as the ability to store intact IGF-I for extended periods (3). Although a role for the capillary endothelium in modulating the delivery of bloodborne IGF-I to different tissues has not been established, the possibility exists that it may be part of a complex regulatory system to control differentially the amount of IGF-I available for binding to receptors on specific target tissues. A similar function has already been proposed for some forms of the IGF-I serum binding proteins (13). If this type of regulatory system operates, it would likely be very important during bGH-stimulated lactation because of the elevated serum IGF-I concentrations. Furthermore, control of mammary specific stimulation of growth by bloodborne IGF-I during bGH treatment could be explained by this type of regulatory system. Alternatively, specific cells could modulate their responsiveness to increased IGF-I in the extracellular environment by simply altering the concentration of IGF-I receptors on their cell surface.

A model whereby bGH stimulates IGF-I production in only nonmammary tissues to influence indirectly the growth or function of mammary epithelial cells appears overly simplistic. The adaptations required for increased milk production in response to bGH probably involve actions of bGH and IGF-I as well as other unidentified hormones and growth factors on both mammary and nonmammary tissues. The results of this study

provide evidence suggesting that IGF-I mediates the action of bGH on at least mammary epithelial cells. This finding offers a possible mechanism for bGH stimulation of lactation. Our results also suggest that IGF-I may be involved in regulating the growth or metabolism of other mammary cell types important in mammary gland function. Whether local IGF-I synthesis occurs in mammary tissue and is stimulated by bGH could not be determined from the results of the present study. Studies are currently underway to investigate this possibility.

Figure II-1. Effect of a single injection of bovine growth hormone (bGH) on serum insulin-like growth factor-I concentration. 20.6 mg bGH were administered subcutaneously at time 0 (arrow). Data points are means for four cows.

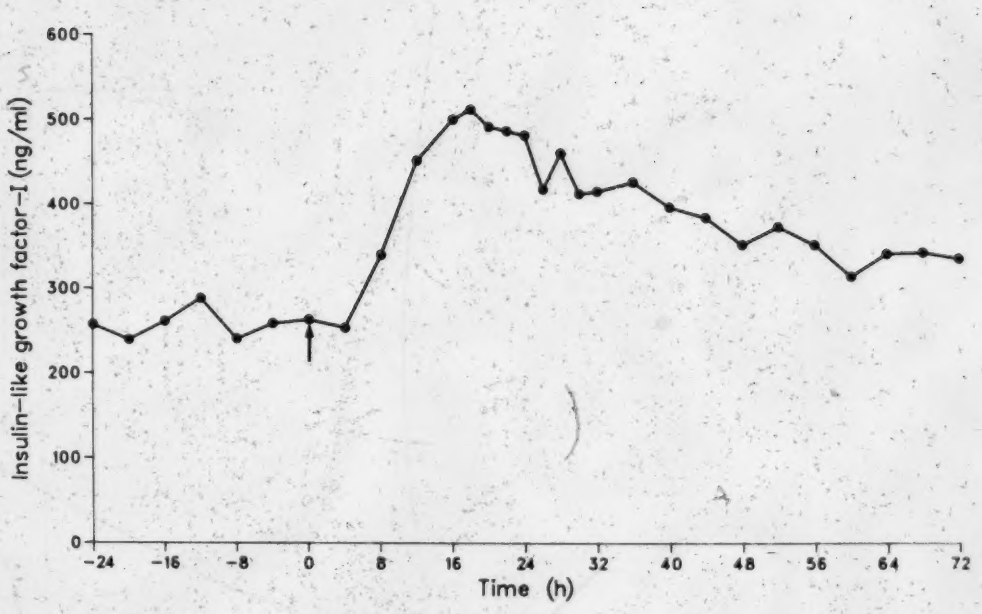

Insulin-like growth factor-I (ng/ml)
600
500
400
300
200
100
0
-24
-16
-8
0
8
16
24
32
40
48
56
64
72
Time (h)

Figure II-2. Effect of three consecutive daily bovine growth hormone (bGH) injections on serum insulin-like growth factor-I concentration. The bGH (20.6 mg/d) was administered subcutaneously (arrows). Mammary biopsies (arrowheads) were taken before and after the bGH treatment period. Data points are means for two cows.

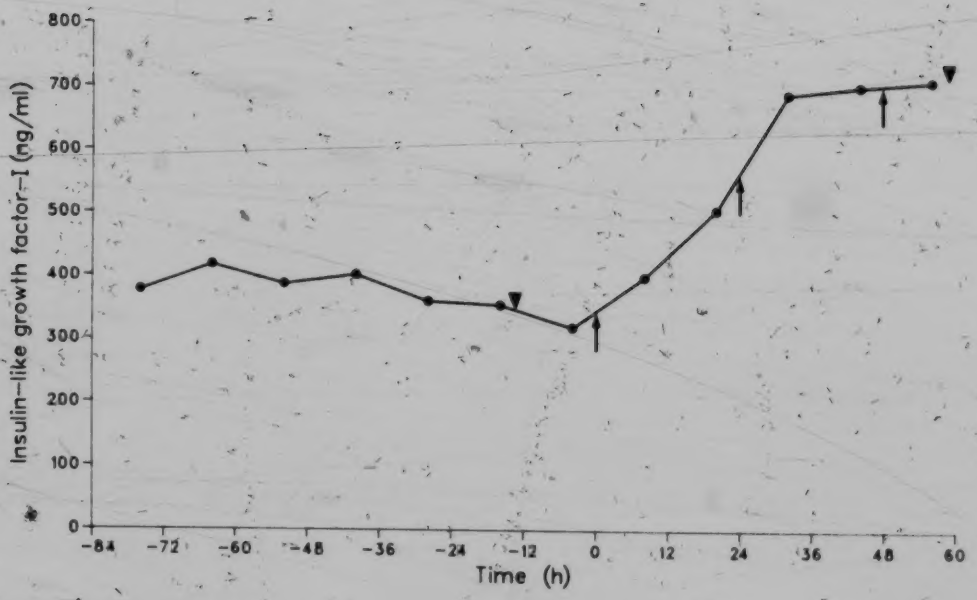

Insulin–like growth factor–I (ng/ml)
800
700
600
500
400
300
200
100
0
-84
-72
-60
-48
-36
-24
-12
0
12
24
36
48
60
Time (h)

Figure II-3. Representative light micrograph of cryostat section from biopsy of lactating bovine mammary tissue stained with hematoxylin and eosin. Ep, alveolar epithelial cells; My, myoepithelial cells; G, fat globules; S, stroma; F, fibroblast-like cells; BV, blood vessels; Cap, capillaries. Bar represents 10 μm.

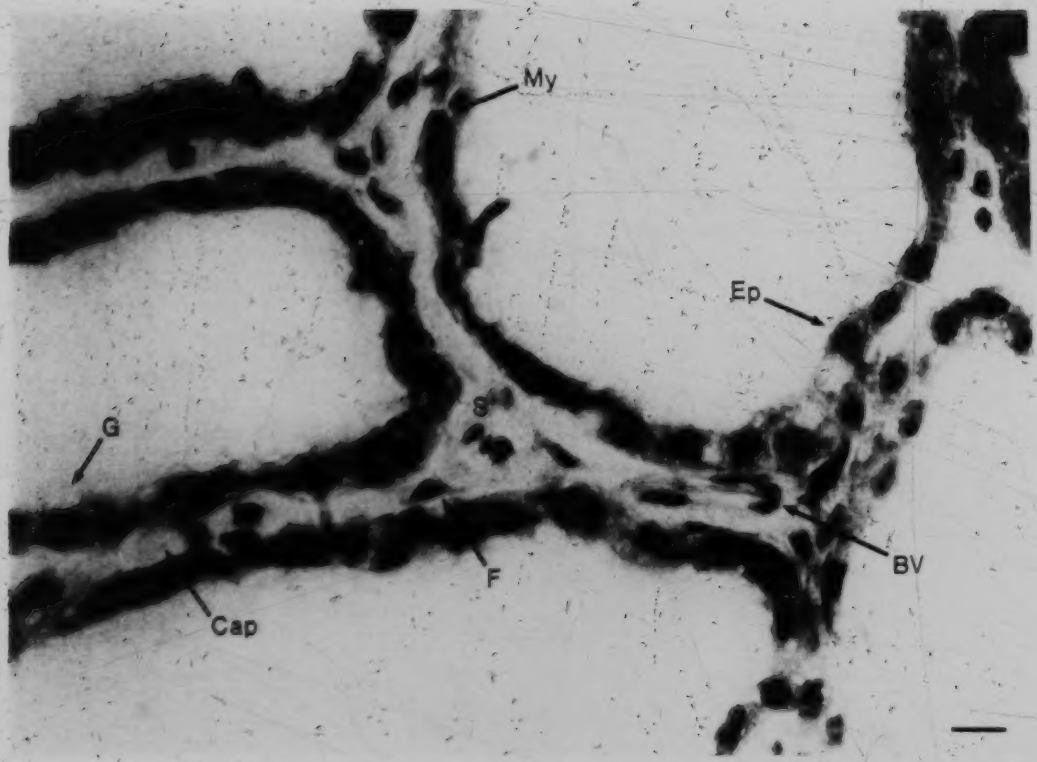

My
Ep
G
S
F
BV
Cap

Figure II-4. Representative immunofluorescent micrographs of insulin-like growth factor-I (IGF-I) localization in mammary tissue from saline treated (control) lactating Holstein cows. Cryostat sections 8-μm thick were stained with 1:1000 dilution of anti-IGF-I antiserum (K1792). A) Tissue from control animal illustrates IGF-I immunoreactivity primarily associated with stromal elements. B) At higher magnification, intensely IGF-I immunoreactive blood vessels (arrow) and unidentified stromal cells (arrowhead) are observed. Note that the alveolar epithelium in control tissue contains only sparse IGF-I-stainable material. Bars represent 10 μm.

A

B

Figure II-5. Representative immunofluorescent micrographs of insulin-like growth factor-I (IGF-I) localization in mammary tissue from bovine growth hormone (bGH)-treated lactating Holstein cows. Cryostat sections 8-μm thick were stained with 1:1000 dilution of anti-IGF-I antiserum (K1792). A) Tissue from bGH-treated animal shows staining of stromal elements (as in control tissue), however there is also staining in the alveolar epithelium. B) At higher magnification, prominent staining is observed in the cytoplasm of epithelial cells (arrows). After bGH treatment, probable myoepithelial cells (arrowhead) are also seen to contain IGF-I-stainable material. Bars represent 10 μm.

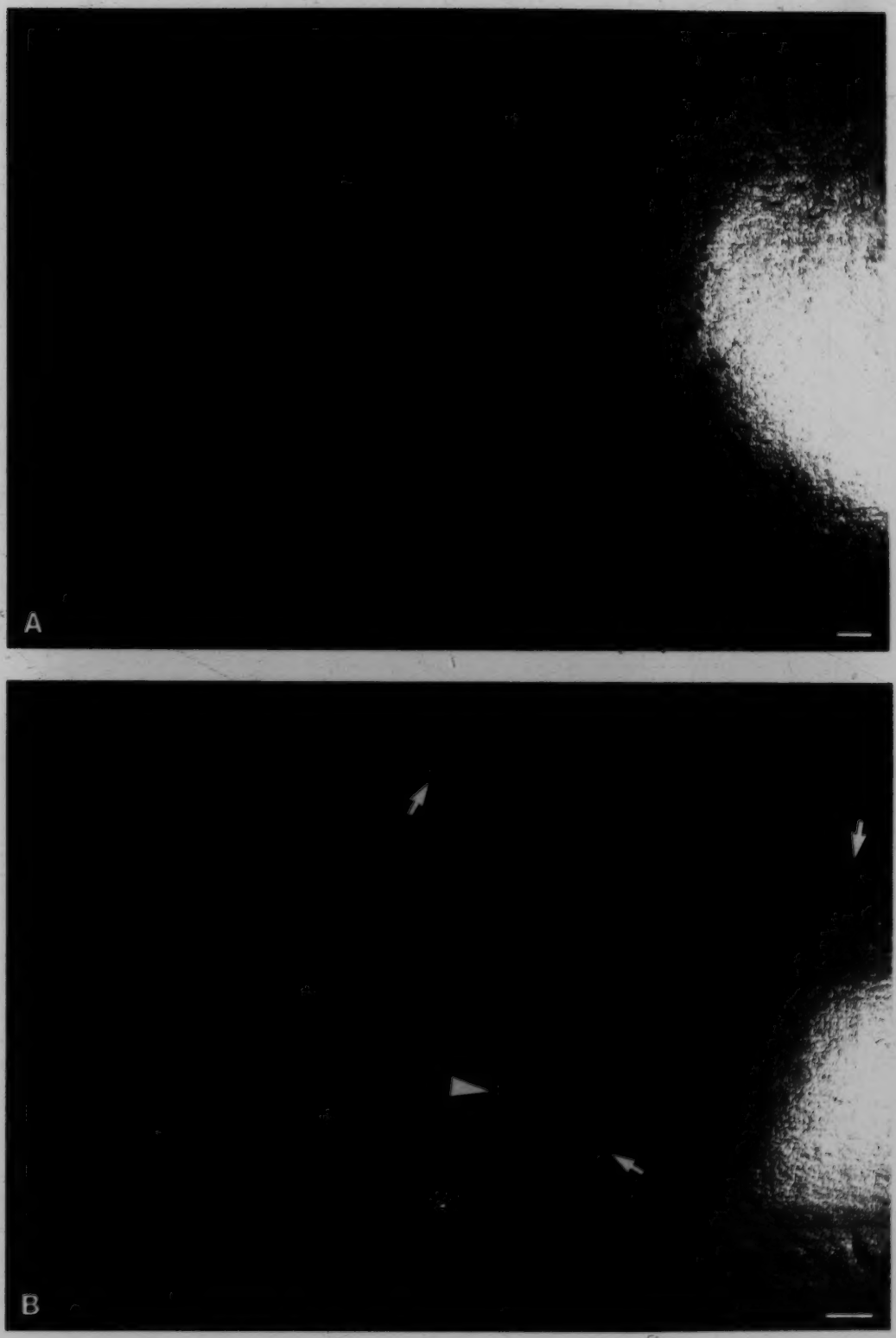
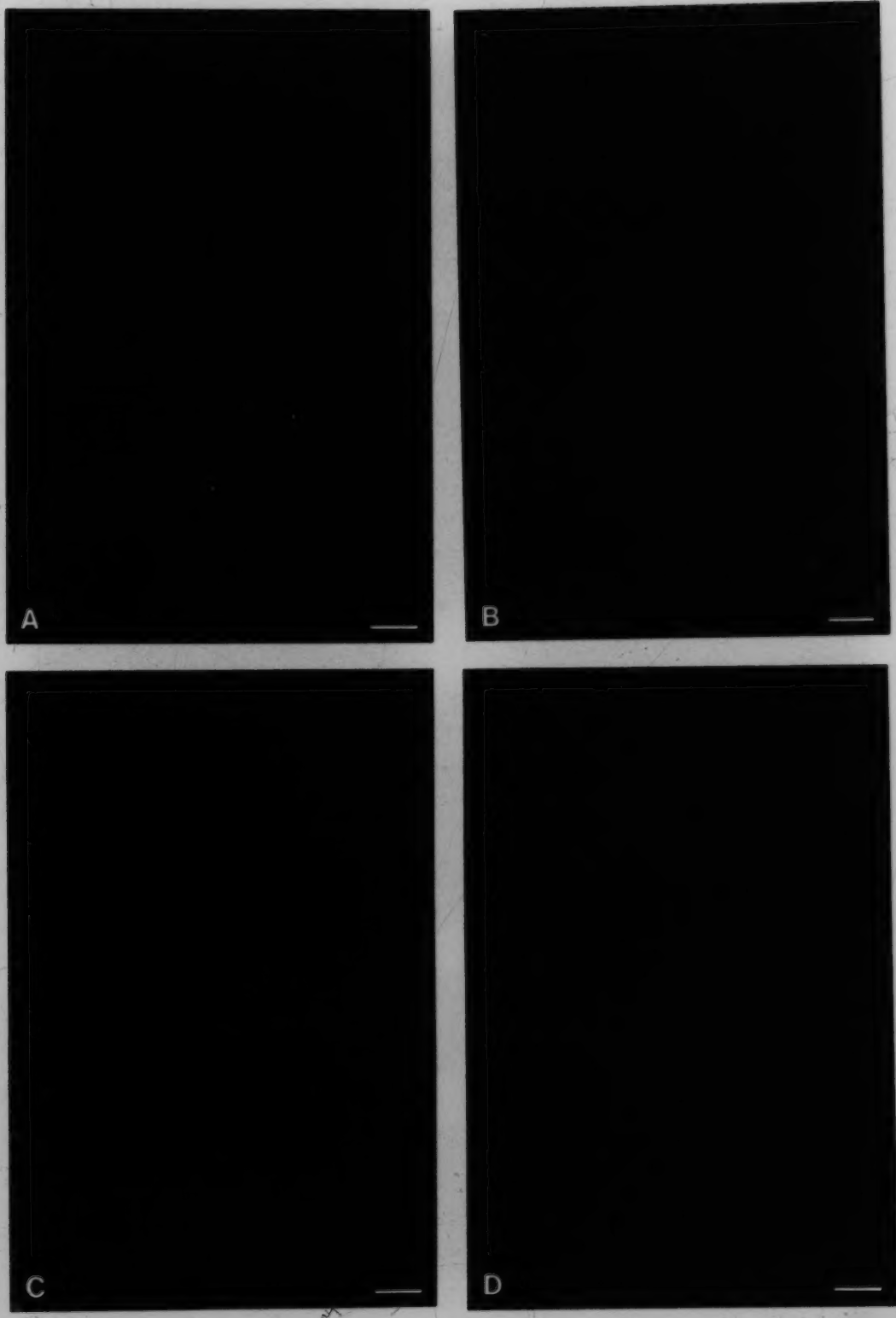

A
B

Figure II-6. Immunofluorescent micrographs of control series demonstrating the specificity of the immunocytochemical reaction. No specific staining is observed in any of the control tests. Bars represent 10 µm. A) Omission of the primary antibody (K1792). B) Omission of the secondary antibody. C) Substitution of the primary antibody with serum from an unimmunized rabbit. D) Incubation with serum depleted of anti-insulin-like growth factor-I antibodies by solid phase immunoabsorption.


A
B
C
D

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Molecular Evidence for the Presence of Growth Hormone Receptors in the Bovine Mammary Gland*

INTRODUCTION

The finding that growth hormone (GH) injection markedly increases milk production in lactating animals has prompted extensive research on the physiological mechanisms involved in this response (2). Although it is likely that GH has effects on various target tissues during both normal and GH-stimulated lactation, these effects and their underlying mechanisms have not been clearly identified. In view of the possibility of widespread GH use in the dairy industry it is important that we understand the mechanism by which GH exerts its stimulatory effect on lactation. Only when the mechanism is established will it be possible to clearly define the implications of long-term GH treatment of dairy cattle.

To exert a physiological effect, it is assumed that GH initially binds with high affinity to receptors at the cell surface of target cells (17). The results of several GH receptor binding studies, using bovine mammary membrane preparations, have led to the conclusion that GH receptors are not present in this tissue (1, 7, 12, 13). It is now widely accepted that GH has direct actions in peripheral tissues and only indirect effects on mammary tissue function. This idea has clearly influenced the direction of research aimed at understanding the mechanism of the response to GH.

Two conceptual problems arise from the apparent absence of GH receptors in mammary tissue. First, it is difficult to reconcile that the marked effects of GH on lactation and *in vivo* mammary growth are exclusively indirect (18). Secondly, many cell types found in mammary tissue, such as fibroblasts, adipocytes, T lymphocytes, and smooth muscle cells have been shown to possess GH receptors in numerous other tissues (10, 17). Thus, it is paradoxical that when mammary membranes are prepared for receptor binding studies, no specific GH binding can be detected, even from these

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* A version of this chapter has been published.
Glimm, D.R., V.E. Baracos, and J.J. Kennelly.
1990. J. Endocrinol. 126:R5-R8.

cell types. Since the preparation of cell membranes for binding studies could conceivably alter or destroy cell surface receptors, we have used molecular hybridization analysis as an alternative approach to examine the question of whether there are GH receptors in mammary tissue.

MATERIALS AND METHODS

Mammary Tissue and RNA Isolation

Mammary tissue was obtained from six lactating Holstein cows immediately after slaughter. Prior to tissue collection, three cows were injected daily for 3 consecutive days with recombinant bovine GH (20.6 mg/d subcutaneous injection; Lot No. PR6776C-169A, American Cyanamid, Princeton, NJ) and the other three cows received no GH injections. Total RNA was isolated from mammary tissue using the guanidine thiocyanate/CsCl procedure (5). RNA was also isolated from rat and rabbit liver using the same procedure. Polyadenylated RNA (poly(A)$^+$ RNA) was isolated from total RNA using oligo(dT) cellulose chromatography (11).

GH Receptor Probe

A 638 base pair rabbit GH receptor cDNA fragment encoding amino acid residues 105-318 of the rabbit GH receptor (16), was subcloned into the plasmid pGEM-3 (Promega, Madison, WI) (20). Radiolabeled sense ([^{35}S]CTP) and antisense ([^{35}S] or [^{32}P]CTP) RNA transcripts were generated from linearized plasmid DNA using T7 or SP6 RNA polymerase respectively (14). Probe specific activities were 7-9 $\times$ 10^8 dpm/μg RNA.

Northern Hybridization

Total RNA was electrophoresed through 1% (w/v) agarose denaturing gels and transferred to nylon membranes. The amount of RNA loaded and transferred was confirmed by UV light visualization of ethidium bromide-stained RNA (6). A .24-9.5 kilobase (kb) RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) was used as a size marker. Membranes were prehybridized for 2 h at 50°C in a buffer consisting of 60% (v/v) formamide, 1$\times$ SSPE (.18 M NaCl, .01 M sodium phosphate (pH 7.4), 1 mM EDTA), .5% (w/v) blotto (low fat milk powder; Carnation Ltd., Toronto, ON), 10% (w/v)

dextran sulphate, 1% (w/v) sodium dodecyl sulphate (SDS) and 500 μg denatured salmon testes DNA/ml. Following hybridization for 18 h at 50°C in fresh buffer containing radiolabeled antisense RNA probe (2×10^6 dpm/ml), membranes were briefly rinsed in 2X SSC (1X SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0), washed for 15 min at 22°C in 2X SSC containing .1% SDS, washed for 15 min at 70°C in .2X SSC containing 1% SDS, and then briefly rinsed in 0.2X SSC. Autoradiography was performed at -70°C.

***In Situ* Hybridization**

Excised mammary tissue pieces were fixed for 6 h at 4°C in freshly prepared 2% (w/v) paraformaldehyde/1% (w/v) glutaraldehyde in phosphate-buffered saline (pH 7.4). Cryostat sections (10 μm) were thaw-mounted onto gelatin-coated slides, and then pretreated, prehybridized, hybridized, and washed as described (9), with some modifications. In brief, deproteinization was carried out for 7.5 min using 20 μg proteinase K/ml (Sigma Chemical Company, St. Louis, MO); sections were not treated with HCl. After postfixation and dehydration, sections were prehybridized for 1 h at 43°C and then hybridized for 18 h at 43°C with approximately 5×10^6 dpm of sense or antisense RNA probe in 50 μl hybridization buffer. Prehybridization and hybridization buffer contained 50% (v/v) formamide, 25 mM Pipes buffer (pH 6.8) with .75 M NaCl and 25 mM EDTA, 5× Denhardt's solution (.1% (w/v) Ficoll 400, .1% (w/v) bovine serum albumin, .1% (w/v) polyvinylpyrrolidone), .2% (w/v) SDS, .1 M dithiothreitol, 250 μg yeast tRNA/ml, and 250 μg denatured salmon testes DNA/ml. After hybridization, washing, and ribonuclease A (RNase A, 50 μg/ml)(Sigma Chemical Company) digestion for 30 min at 37°C, sections were air dried and then dipped in NTB-2 photographic emulsion (Eastman Kodak, Rochester, NY). Sections were exposed for 12 d, developed, stained with hematoxylin, and analysed using brightfield and darkfield microscopy.

RESULTS

Two GH receptor transcripts of 4.4 and 9.2 kb were detected in mammary total RNA from both normal and GH-treated lactating cows (Figure III-1). The abundance of both transcripts appeared to be lower in RNA from two GH-treated animals than that

seen in RNA from normal animals. No hybridization to ribosomal RNA was detected in mammary total RNA depleted of poly(A)$^+$ RNA by three rounds of oligo(dT) chromatography. The GH receptor probe detected two transcripts with similar abundance of 4.2 and 1.3 kb in rat liver total RNA, and a major transcript of 4.2 kb in rabbit liver total RNA (Figure III-1). Two less abundant transcripts of 12.5 and 6.6 kb were also detected in rabbit liver RNA.

GH receptor gene expression in normal bovine mammary tissue was primarily localized to the alveolar epithelial cell region (Figure III-2A). Cells located in the stromal matrix showed a lower level of detectable hybridization. The distribution of silver grains observed in mammary tissue sections obtained from GH-treated cows was similar to that seen in normal tissue (data not shown). Tissue sections that were hybridized with a radiolabeled GH receptor sense RNA probe showed no positive localization above background in any region (Figure III-2B). RNase A pretreatment (100 μg/ml for 15 min) of the tissue sections before hybridization also resulted in no signal above background (data not shown). These observations suggest that we are observing the proper, complementary hybrids of the antisense RNAs with the corresponding GH receptor mRNAs.

DISCUSSION

This study identified and characterized GH receptor mRNAs in lactating bovine mammary tissue using Northern and *in situ* hybridization analyses. We found that mammary total RNA from both normal and GH-treated cows contained two GH receptor mRNA species. Further, the abundance of GH receptor transcripts appeared lower in mammary tissue from two GH-treated cows compared with all normal cows. Although additional research using more animals is required to confirm this finding, it suggests that GH receptor down-regulation may occur in mammary tissue during GH treatment, and be regulated at the level of transcription or mRNA turnover. In both normal and GH-treated cows, alveolar epithelial cells were shown to be the major cell type in mammary tissue that express the GH receptor gene. In contrast to GH receptor binding

studies, these observations provide evidence that the bovine mammary gland is a GH target tissue.

The different sized GH receptor mRNAs present in mammary tissue could be derived from the same gene either by use of different initiation or polyadenylation sites, or by alternative splicing of a common primary transcript (15). The different mRNAs could also simply represent an accumulation of partially spliced molecules with different combinations of persisting introns or, alternatively, they could represent transcripts from distinct genes (4). Although a GH binding protein has not been identified in bovine serum, it is noteworthy that the mRNA coding for the rat GH serum binding protein is probably an alternatively spliced form of GH receptor mRNA (3).

Using the GH receptor RNA probe, we also detected two GH receptor mRNAs with similar abundance in rat liver of 4.2 and 1.3 kb. These mRNAs correspond to the two transcripts with similar abundance previously detected in rat liver using a rat GH receptor cDNA probe (3). We detected one major GH receptor transcript of 4.2 kb and two additional less abundant transcripts of 12.5 and 6.6 kb in rabbit liver. Two separate studies have reported similar sizes for the single major GH receptor mRNA present in rabbit liver (16, 17). Two GH receptor mRNAs of similar abundance and size have also been found in mouse liver and adipose tissue (19).

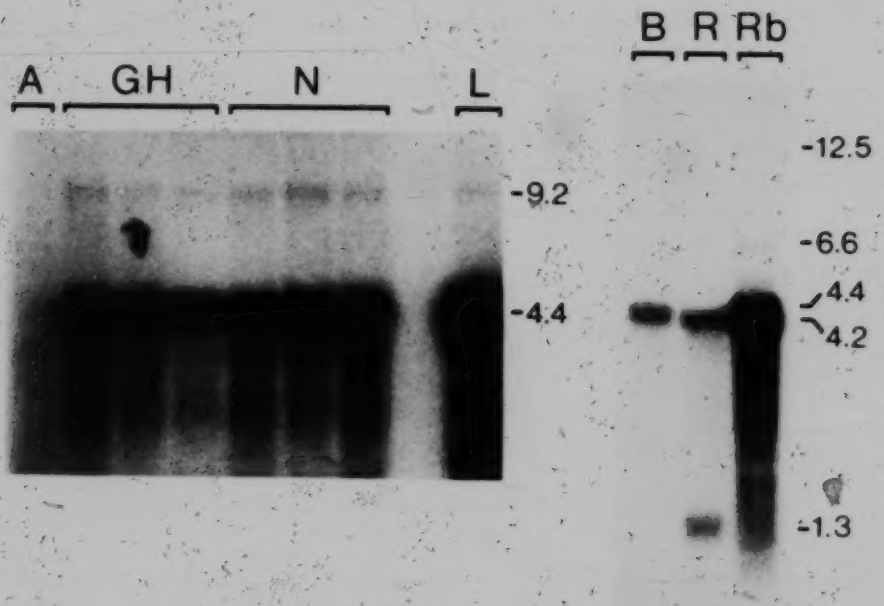
In situ hybridization analysis using mammary tissue sections revealed that the alveolar epithelial cells are the primary site of GH receptor gene expression in the lactating mammary gland of cows. Interestingly, insulin-like growth factor-I (IGF-I) and homologous receptor (type I) mRNAs are also present in this tissues during lactation (unpublished observations). It is therefore conceivable that GH interacts directly with alveolar epithelial cells to induce the production of IGF-I, which in turn may regulate their growth or function through an autocrine or paracrine mechanism.

The lower hybridization signal observed over cells located in the stromal region suggests that during lactation these cells either express the GH receptor gene at a lower rate, or that GH receptor mRNA is less stable in these cells. Considering the importance of mammary stromal-epithelial interactions, particularly during

mammeogenesis (8), a detailed analysis of GH receptor mRNA structure and function in the cells of both these tissue regions may provide insight into mechanisms controlling mammary growth and function.

The existence of GH receptor mRNAs in bovine mammary tissue suggests that specific mammary cells possess GH receptors. The alternative, that this receptor gene is transcribed in mammary tissue but its RNA is not translated, is unlikely. Our results therefore provide strong evidence that mammary tissue is a GH target tissue, with capacity for biological responsiveness to GH during lactation. This finding challenges the widely accepted view that GH does not directly influence mammary function and it provides a basis for future research aimed at understanding the mechanism by which GH exerts its stimulatory effect on lactation.

Figure III-1. Northern hybridization analysis of total RNA probed with radiolabeled GH receptor antisense RNA. *Left panel*: mammary RNA from GH-treated (GH, 30 μg) and normal (N, 30 μg) lactating Holstein cows. Mammary total RNA depleted of poly(A)$^+$ RNA (A, 30 μg) and bovine liver RNA (L, 10 μg) are also shown. *Right panel*: RNA from bovine (B, 5 μg), rat (R, 5 μg), and rabbit (Rb, 5 μg) liver. Band sizes are indicated in kilobases at the right of each panel.


A
GH
N
L
-9.2
-4.4
B R Rb
-12.5
-6.6
4.4
4.2
-1.3

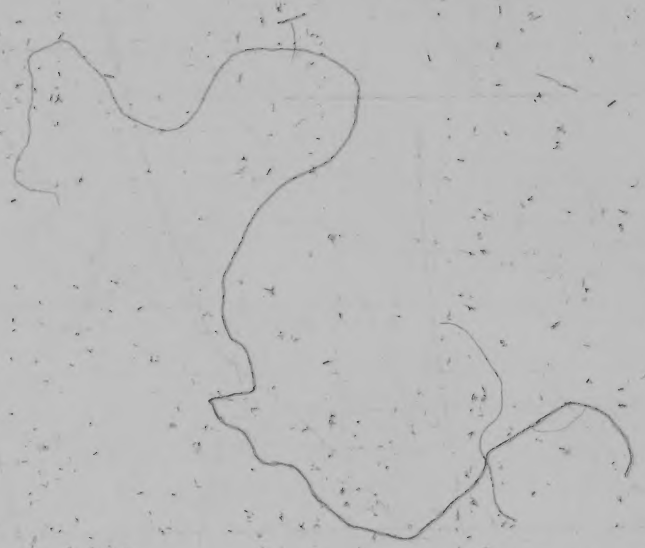
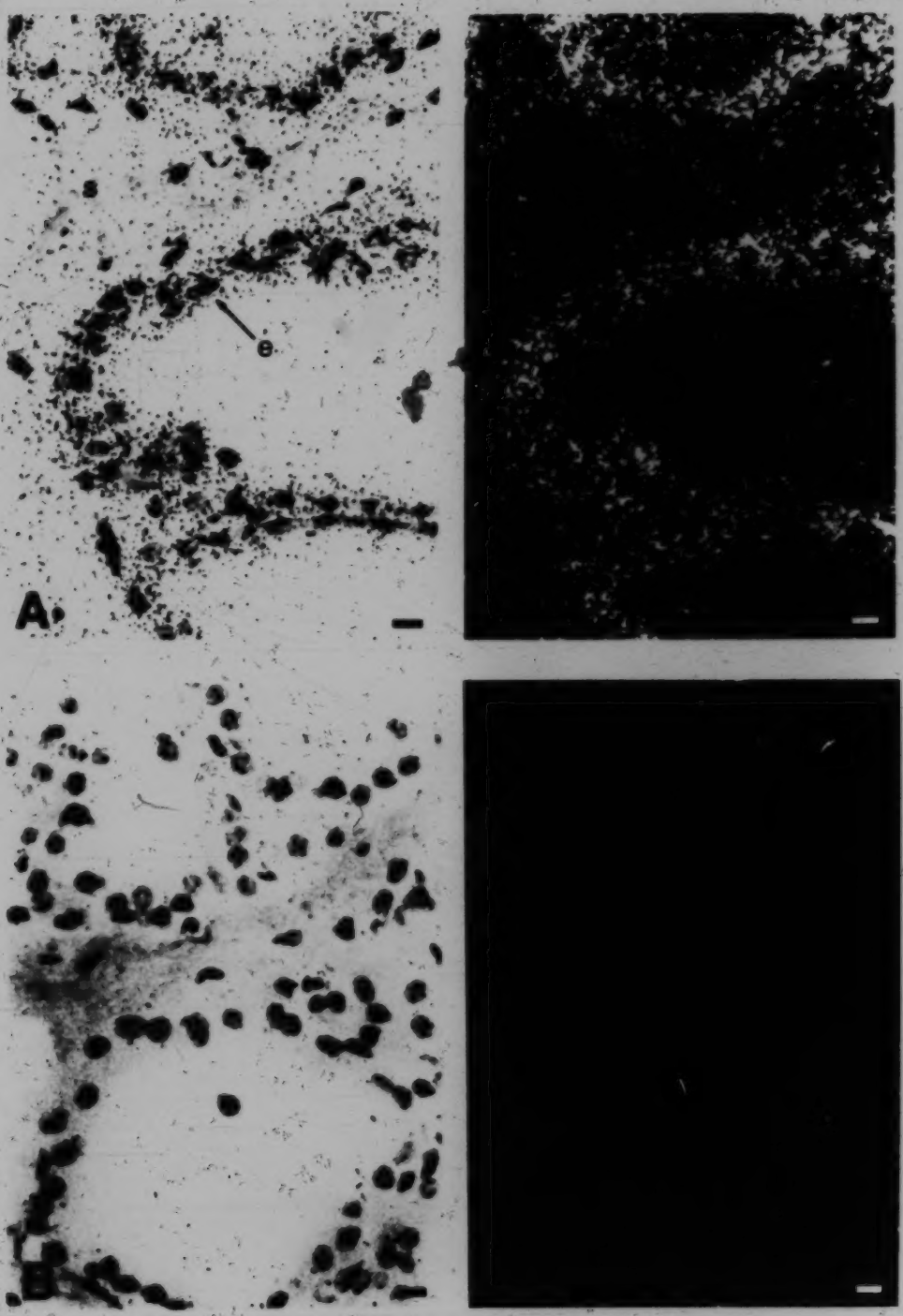


Figure III-2. *In situ* hybridization of radiolabeled GH receptor (A) antisense and (B) sense RNA probes to sections of normal lactating bovine mammary tissue. The right panels are darkfield images of the same section location shown in brightfield illumination in the left panels. Examples of alveolar epithelial cells (e) and stroma (s) are indicated. Bars represent 10 μm.


e
A
B

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Growth Hormone Regulation of Insulin-like Growth Factor-I, Type I Insulin-like Growth Factor Receptor, and Epidermal Growth Factor Receptor Gene Expression in Mammary Tissue of Lactating Dairy Cows

INTRODUCTION

Of the many hormones involved in the regulation of lactation, growth hormone (GH) is considered to play a key role because of the marked increase in milk production that occurs when lactating animals are given GH injections (5, 63). Increased milk synthesis must reflect direct or indirect GH action on mammary cell function. Until recently, direct GH action on mammary cells was considered unlikely because several GH receptor binding studies suggested that mammary cells did not possess GH receptors (1, 26, 43, 44). Based on this information it has been widely accepted that GH induces milk synthesis primarily through the partitioning of nutrients to the mammary gland (3, 4, 7, 64, 65). Although it is likely that such metabolic effects are an important part of the response to GH, alone they do not adequately explain the mechanism of GH action.

Recently, GH receptor mRNA analysis experiments have provided evidence that the alveolar epithelial cells may indeed synthesize functional GH receptors, and also revealed that GH injection may cause mammary GH receptors to be down regulated (30). These findings clearly raise the possibility of direct GH action on mammary cells.

The changes in the mammary gland required for increased milk synthesis have not been clearly identified. An experiment using pairs of monozygotic twin heifers revealed that GH injection increased mammary parenchymal proliferation (76). Whether this response also occurs in lactating animals has not been investigated, but it has been shown that mammary growth can occur during established lactation (22, 42, 46, 47, 95). The results of *in vitro* studies however, have shown that adding GH to cultured lactating mammary explants does not stimulate cellular proliferation (6, 27), and generally does not alter either casein or α-lactalbumin synthesis or secretion (27, 31). A few *in vitro* experiments have detected changes in the production of some milk constituents after GH addition, but the changes have either been small or considered

the result of prolactin contamination in GH preparations (61, 81). By contrast, GH injection of athymic mice implanted with bovine mammary tissue explants results in substantial accumulation of α-lactalbumin, as well as growth and differentiation in the implanted tissue (79).

An important open question in the understanding of GH action on the mammary gland is how GH effects the expression of specific genes coding for regulatory molecules of potential importance in the control of mammary growth and function. The most likely candidate for this role is insulin-like growth factor-I (IGF-I), which is believed to mediate GH actions on various tissues in other species (35, 75). We have presented immunocytochemical evidence that supports a role for IGF-I in inducing mammary changes in response to GH injection (29). After GH treatment, an accumulation of IGF-I immunoreactivity is observed in lactating alveolar epithelial cells. These cells therefore either internalize or synthesize IGF-I in response to GH treatment. Receptor binding studies have demonstrated that mammary epithelial cells possess type I IGF receptors (10, 17, 32). Thus, they possess the ability to internalize IGF-I. The possibility that IGF-I is synthesized by epithelial cells during GH treatment is supported by the finding that cultured human breast cancer cells synthesize and secrete IGF-I (23, 36).

Several different *in vitro* systems, using mammary tissue explants or isolated cells from different species, have recently been used to examine the effects of IGF-I on the growth and function of mammary cells (24, 38, 50, 67). A general conclusion of these studies is that IGF-I is a potent mitogen for both transformed and normal mammary cells. IGF-I apparently also plays a role in the control of carrier-mediated glucose transport in mammary cells (66, 67).

Another potent mitogen for mammary alveolar epithelial cells is epidermal growth factor (EGF) (39, 84, 88). Proliferative phases of mammary growth are paralleled by the cell surface complement of EGF receptors on epithelial cells (20). However, EGF also inhibits the functional differentiation of epithelial cells, by

decreasing their responsiveness to stimulators of casein and α-lactalbumin synthesis (84). Thus, EGF is a multifunctional regulator of mammary physiology.

In the present study, we used RNA analysis methodology to investigate the effects of GH injection on the mammary tissue expression of genes coding for IGF-I, type I IGF receptor, and EGF receptor. In the course of these studies, efforts were also devoted to the development of technical conditions that allowed the efficient and reproducible detection of mRNA in mammary tissue.

MATERIALS AND METHODS

Animals and Experimental Design

Six Holstein cows in second or later lactation were used. Animals were fed a complete mixed diet containing 50% concentrate and 50% forage. Three animals received daily saline injections (2.0 ml, .9% NaCl; subcutaneous injection) for 3 consecutive d and then bGH (20.6 mg/d; subcutaneous injection; Lot No. PR6776C-169A; American Cyanamid, Princeton, NJ) injections for another 3 consecutive d. The other three animals received no injections. Mammary tissue was sampled from injected cows by percutaneous biopsy 10 h after both the last saline and GH injections; mammary tissue was also obtained from all cows immediately after slaughter. Animals were milked 3 to 4 h prior to mammary tissue collection.

Mammary Tissue Handling

Immediately after removal, mammary tissue pieces were either rapidly frozen in liquid nitrogen or processed for *in situ* hybridization. Tissue for RNA isolation was stored at -70°C. Tissue for *in situ* hybridization was fixed for 6 h at 4°C in freshly prepared 2% paraformaldehyde (w/v)/1% glutaraldehyde (w/v) in phosphate-buffered saline (PBS, pH 7.4). Following fixation, tissue was washed with three changes of PBS, 30 min each, at 4°C. Tissue was then cryoprotected by immersion in 30% sucrose (wt/vol) in PBS for 18 h at 4°C. Tissue was mounted in OTC compound (Ames Company, Elkhart, IN) by rapidly freezing in isopentane (-150°C) cooled by liquid nitrogen. Mounted tissue was stored in air tight polypropylene bags at -70°C.

Total RNA Isolation

Three different total RNA isolation procedures were evaluated. The first procedure involved tissue homogenization in a lysis buffer, followed by repeated phenol/chloroform extractions, and finally ethanol precipitation of RNA (28). This procedure was labor intensive and resulted in variable and incomplete recovery of RNA from mammary tissue (yields ranged from .8 mg/g to 2.1 mg/g tissue). The purity of the RNA obtained by this procedure was also variable and frequently unacceptable. In the second procedure, tissue was homogenized in a guanidine isothiocyante (GIT) solution, repeatedly extracted with phenol/chloroform, and then RNA was ethanol precipitated (14). Although the purity of RNA obtained by this method was similar to that obtained by the GIT/CsCl method (see below), its use resulted in lower yields of RNA. In the third procedure (GIT/CsCl) (13), sample processing was simple and rapid, RNA yields were reproducible (2.5 ± .3 mg/g tissue), and the UV light absorbance ratio (260 nm/280 nm) of RNA consistently ranged from 1.95 to 2.05. This procedure was therefore used routinely.

In the GIT/CsCl procedure, frozen tissue (.9 to 1.0 g) was pulverized in liquid nitrogen using a prechilled (-70°C) mortar and pestle. Frozen, pulverized tissue was then immediately transferred to a 30 ml Corex tube containing 9.0 ml of GIT solution [4.0 M GIT, 25 mM Tris-HCl (pH 7.5), .5% sodium N-lauryl sarcosine (wt/vol), .1 M β-mercaptoethanol]. The pH of the GIT solution was not adjusted. The tissue was homogenized using a Polytron at full speed for 60 sec, at room temperature. To remove tissue debris, the homogenate was centrifuged at 5,000 *g* for 10 min, at room temperature. The supernatant was layered onto 3.3 ml of CsCl solution [5.7 M CsCl, .1 M disodium ethylenediaminetetra acetic acid (EDTA), pH 7.0] in a polyallomer ultracentrifugation tube (Cat. No. 331372; Beckman Canada, Burnaby, BC). Before use, polyallomer tubes were filled with .1 NaOH, allowed to stand for 5 min, and then thoroughly rinsed with sterile, deionized H_20. Tubes were ultracentrifuged in a SW 41 Ti rotor (Beckman Canada) at 30,000 rpm for 23 h (20°C).

After ultracentrifugation, the tissue homogenate and CsCl solutions were aspirated. RNA pellets were dissolved in SET buffer containing 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and .1% sodium dodecyl sulfate (SDS; wt/vol) by heating at 60°C for 5 min and then vortexing. RNA was stored at -70°C. RNA was also isolated from bovine, rat, and rabbit liver, and human placenta using this procedure. Total RNA was isolated from biopsy tissue and small tissue samples (10 to 150 mg) using a scaled-down version of the GIT/CsCl procedure; ultracentrifugation was performed at 42,000 rpm for 12 h using a SW 50.1 rotor (Beckman, Canada).

Polyadenylated RNA Isolation

Two procedures were evaluated for their usefulness in isolation of polyadenylated [poly(A)$^+$] RNA from total RNA. Both procedures involve chromatography using oligo d(T)-cellulose (Type 3; Collaborative Research, Bedford, MA). A procedure using NaCl-based buffers was performed essentially as described (40). Using this procedure, the sodium salt of LiCl frequently precipitated in NaCl buffers and impeded column flow. This resulted in unacceptable variation in yields of poly(A)$^+$ RNA ranging from 2.7 to 7.8% of input total RNA.

A procedure substituting NaCl-based with LiCl-based buffers (72) was also used. Column flow was not impeded when LiCl buffers were used; poly(A)$^+$ RNA yields ranged from 1.2 to 1.5% of input total RNA after two rounds of oligo d(T) chromatography using LiCl buffers. This procedure was therefore used for all poly(A)$^+$ RNA isolations. In this procedure, .2 g of oligo d(T) was used for every 5.0 mg of total RNA. Oligo d(T) was suspended in EB; 1 ml EB was used for each .1 g oligo d(T). The suspension was gently agitated and allowed to stand for 1 to 3 min. Fine particles of oligo d(T) were then removed by aspirating the EB. This process of removing fine particles was repeated 3 times. The oligo d(T) slurry was poured into an autoclaved, disposable polypropylene column (Cat. No. 731-1550, Bio-Rad Canada, Mississauga, ON) and washed with ten column volumes of binding buffer (BB).

RNA (2 mg/ml) in SET buffer was heated at 65°C for 10 min and 10 M LiCl was added to make the solution .5 M LiCl. The RNA solution was loaded onto the

column and the effluent collected and saved. The column was then washed with five column volumes of BB. The original effluent was passed through the column a second time and again washed with BB. This procedure was repeated one more time. The column was then washed with two column volumes of wash buffer (WB). Poly(A)$^+$ RNA was eluted using two column volumes of EB.

The poly(A)$^+$ RNA enriched solution was then subjected to each step of the aforementioned chromatography procedure, except that a pre-equilibrated column with less oligo d(T) was used. For every 10 mg of input total RNA, .1 g of oligo d(T) was used for the second column; fine particles were removed before pouring the column. Poly(A)$^+$ RNA was ethanol precipitated from the final eluate, dissolved in SET, and stored at -70°C.

RNA Gel Electrophoresis

RNA was electrophoresed through 1% agarose (wt/vol) gels containing .66 M formaldehyde and morpholinopropanesulfonic acid (MOPS) running buffer (.02 M MOPS, 5 mM sodium acetate, 1mM EDTA, pH 7.0). RNA samples (up to 30 µg in 5 µl) were incubated at 65°C for 15 min with 25 µl loading buffer [.02 M MOPS, 50% deionized formamide (vol/vol), 1.3% glycerol (vol/vol), .1% bromophenol blue (wt/vol), 2.0 M formaldehyde]. After briefly chilling RNA samples on ice, 1 µl of 1.0 mg/ml ethidium bromide was added to each, and then RNA samples were loaded onto a gel. A 24-9.5 kilobase (kb) RNA ladder (Bethesda Research Laboratories, Gaithersberg, MD) was used as a size marker and treated exactly as RNA samples. Gels were run for 10 h at 40V (constant voltage) with continuous buffer recirculation.

Northern Transfer

Gels were photographed on a UV transilluminator after electrophoresis and then soaked in two changes (20 min each) of 10× SSC (1× SSC is .15 M NaCl, .015 M trisodium citrate, pH 7.0). RNA was transferred to nylon membranes (Zeta-Probe; Bio-Rad Canada) by capillary transfer using the sponge configuration and 10× SSC as the transfer solution (25). After transfer (10-12 h), membranes were photographed on a UV transilluminator, allowed to dry for 30 min at room temperature, and baked in a

vacuum oven for 2 h (80°C). Fixation of RNA to nylon membranes by baking at 80°C resulted in consistent signal intensities between similar hybridizations. The extent of RNA fixation to membranes using UV light was difficult to control, and therefore not reproducible. Baked membranes were stored in heat-sealed plastic bags at 4°C.

Radiolabeled Riboprobes

Radiolabeled IGF-I antisense riboprobes ([^{32}P] or [^{35}S]CTP) were generated from Pvu II or Avi II linearized plasmid DNA [pGEM-1/hIGF-IB (71)] using SP6 RNA polymerase (48). Radiolabeled IGF-I sense riboprobes ([^{35}S]CTP) were generated using Bam HI linearized plasmid DNA and T7 RNA polymerase. IGF-I sense and antisense riboprobe specific activities were 1.6-1.9 × 10^9 dpm/μg RNA.

A 2700 base pair (bp) human type I IGF receptor cDNA fragment (89) was subcloned into the plasmid pGEM-3 (Promega, Madison, WI) (83). Radiolabeled type I IGF receptor antisense riboprobes ([^{32}P] or [^{35}S]CTP) were generated from Eco RV linearized plasmid DNA using T7 RNA polymerase. Radiolabeled sense riboprobes ([^{35}S]CTP) were generated from Pvu II linearized plasmid DNA using SP6 RNA polymerase. Type I IGF receptor antisense and sense riboprobe specific activities were 1.6-1.8 × 10^9 dpm/μg RNA.

EGF receptor antisense ([^{32}P] or [^{35}S]CTP) and sense ([^{35}S]CTP) riboprobes were generated from Bam HI and Hind III linearized plasmid respectively using either SP6 (antisense) or T7 (sense) RNA polymerase; the EGF receptor plasmid (pGEM-3, Promega) contained a human EGF receptor cDNA fragment (694 bp) derived from pE7 (94), which was obtained from American Type Culture Collection. EGF receptor sense and antisense riboprobe specific activities were 1.6-1.7 × 10^9 dpm/μg RNA.

GH receptor antisense riboprobes ([^{32}P]CTP) were generated using T7 RNA polymerase as described (30). Probe specific activities were 1.1-1.4 × 10^9 dpm/μg RNA.

After labeling riboprobes, template DNA was digested with RQ 1 DNase (37°C, 15 min; Promega). Riboprobes were then extracted with Tris-buffered phenol/chloroform, chloroform/isoamylalcohol (24:1), and unincorporated, radiolabeled CTP was removed by

Sephadex G-50 (DNA grade, fine; Pharmacia Canada, Dorval, PQ) spun column chromatography (8).

Northern Hybridization

Membranes were prehybridized, hybridized using antisense riboprobes at 2 × 10^6 dpm/ml hybridization solution, and then washed as described (30). Some membranes were subjected to stringency tests by either an elevated wash temperature (75 or 80°C instead of 70°C), or digestion with ribonuclease A (RNase A; Sigma Chemical Company, St. Louis, MO) after the normal wash protocol. RNase A (10 µg/ml) digestion was performed in 2× SSC for 15 min at 37°C. After digestion, membranes were washed in .2× SSC/1% SDS (wt/vol) at 50°C for 15 min and finally rinsed in .2× SSC. Autoradiography of Northern blots was performed at -70°C using intensifying screens (Dupont Canada, Mississauga, ON).

Alkaline Hydrolysis of Riboprobes

Shorter riboprobes were tested for their usefulness in *in situ* hybridization. After transcription labeling, riboprobes were hydrolyzed to approximately 150 bp under alkaline conditions as described (2). Following neutralization, riboprobes were purified by Sephadex G-50 spun column chromatography. Hydrolyzed transcript size was confirmed using gel electrophoresis, followed by fluorography/autoradiography with Enlightening autoradiographic enhancer (Dupont Canada).

***In Situ* Hybridization**

Tissue sectioning and pretreatment. OTC-mounted tissue was removed from -70°C and allowed to equilibrate to -20°C for 2 h before cryosectioning. Tissue sections were cut at 10 µm in a cryostat (Reichert-Jung, Nussloch, West Germany) at -20°C and thaw-mounted onto chrom-alum coated slides (30 min, room temperature). Slides with tissues sections were stored at 4°C for up to 2 d before performing *in situ* hybridization.

Tissue sections were pretreated as described (34), with some modifications. Deproteinization was carried out for 7.5 min using 20 µg/ml proteinase K (Sigma Chemical Company); sections were not treated with HCl. Some sections were not pretreated; however, non-pretreated sections were prefixed in 4% paraformaldehyde

(wt/vol) PBS for 5 min, dehydrated in an accending ethanol series, and air dried for 30 min before prehybridization.

Prehybridization and hybridization of tissue sections. Prehybridization and hybridization were carried out in *in situ* hybridization chambers (Tyler Research Instruments, Edmonton, AB) as described (34), with some modifications. In brief, sections were prehybridized for 1-2 h at 43°C and then hybridized for 16-18 h at 43°C with approximately 5×10^6 dpm of sense or antisense riboprobe in 50 µl hybridization buffer. The composition of the prehybridization and hybridization buffers have been reported (30). For some *in situ* hybridizations, the prehybridization and hybridization buffers were modified to contain 10% dextran sulfate (wt/vol) (Pharmacia Canada).

After hybridization, washing, and RNase A (50 µg/ml) digestion for 30 min at 37°C, sections were air dried (1 h, room temperature). The intensity of the hybridization signal was estimated by preliminary autoradiography at room temperature using XAR-5 x-ray film (Eastman Kodak Canada, Toronto, ON). Slides were stored at 4°C until performing emulsion autoradiography.

In situ hybridization specificity controls. Several controls were conducted to check the specificity of *in situ* hybridization reactions. The controls included: 1) testing for positive chemographic effects by incubating tissue sections with unlabeled antisense riboprobes, 2) testing for negative chemographic effects by incubating sections with unlabeled antisense riboprobes and then briefly exposing the emulsion-coated slide to light, 3) testing that hybridization efficiency of mRNA accessibility was similar among different cells in mammary tissue by subjecting tissue to different extents of deproteinization, 4) testing higher final washing temperatures (55 and 65°C in .1× SSC for 45 min), 5) pre-incubating and co-incubating with excess unlabeled antisense riboprobes, and 6) hybridization with tissues sections pre-incubated with RNase A (100 µg/ml in PBS for 1 h at 37°C). Riboprobes hybridized on sections pre-incubated with RNase A were recovered after hybridization, subjected to denaturing gel electrophoresis, and then analyzed by fluorography/autoradiography.

Liquid Emulsion Microautoradiography

Emulsion handling and slide coating. In the dark, NTB-2 nuclear track emulsion (Eastman Kodak Canada) was melted at 45°C in a 50 ml polypropylene centrifuge tube (Cat. No. 25330-50; Corning Laboratory Sciences, Richmond Hill, ON) containing an equal volume of prewarmed (45°C) deionized H_20. The diluted emulsion was stirred gently using a blank slide, allowed to stand (waterbath, 45°C) for 30 min, and then surface bubbles were removed by dipping several blank slides. Slides containing hybridized tissue sections were individually dipped, excess emulsion drained by blotting the slide edge on a paper towel (5 sec), and emulsion wiped from the back of the slide with tissue paper. Emulsion coated slides were then placed (horizontal position) on a cooled stainless steel plate (on ice) for 10 min, to gel the emulsion. The slides were placed (horizontal position) in a light-tight box and allowed to dry for 2 h at room temperature (21°C) and humidity (50%). Next, the slides were placed in open slide boxes in a large dessicator containing dessicant and allowed to dry overnight (10-12 h). Slide boxes containing the dry slides and dessicant were wrapped in aluminum foil and sealed in air-tight polypropylene bags, also containing dessicant; slide boxes were not sealed with tape. Slides were exposed at 4°C.

Emulsion development. Boxes containing exposed slides were allowed to warm up at room temperature for 30 min before opening for development. In the dark, slides were transferred to glass slide racks and developed for 2 min in D-19 developer (15°C) (Eastman Kodak Canada). Slides were briefly agitated in developer every 30 sec. Slides were then rinsed for 15 sec in deionized H_20 (15°C), and fixed for 5 min in Kodak fixer (15°C) (Cat No. 123-8146; Eastman Kodak Canada). Finally, slides were rinsed for 5 min in deionized H_20 (15°C) and then for 30 min in running tap water (15°C).

Histological staining and photomicrography. Emulsion coated tissue sections were stained at room temperature for 1 min in hematoxylin (Cat. No. RO 3312-76; BDH), rinsed in H_20 (30 sec), and dehydrated through an accending ethanol series (30 sec each in 30, 60, 80, 95, 100, and 100% ethanol). Tissue was then cleared by two

rinses (2 min each) in xylene, and coverslips were mounted with Permount (Fisher Canada, Edmonton, AB).

Photomicrography was performed using lightfield and darkfield optics. Photographic images were recorded on black and white film (HP-5, 400 ASA; Ilford Canada, Toronto, ON).

RESULTS

Detection of Insulin-like Growth Factor-I mRNA in Mammary Tissue

In order to detect IGF-I mRNA in mammary tissue it was necessary to develop a reproducible poly(A)$^+$ RNA isolation procedure. This was accomplished using oligo d(T) cellulose chromatography and LiCl-based buffers. The effectiveness of the chromatography procedure is illustrated in Figure IV-1 using the GH receptor riboprobe. After passing rat liver total RNA through the column once, the eluate (lane E_1) still contained a detectable amount of GH receptor mRNA. Less GH receptor mRNA was detected in the eluate collected after a second passage (lane E_2). There was no detectable GH receptor mRNA after a third passage (lane E_3; A RNA). Only a small amount of detectable GH receptor mRNA was released from the column as a result of the wash step (lane W); approximately 2% of the input total RNA was eluted during this step.

After eluting the material retained by the column, it was passed through a second column three more times, washed, and again eluted. Rat GH receptor mRNA was easily detected in poly(A)$^+$ RNA obtained from this final eluate (A_3^+). A comparison of the amount of GH receptor mRNA in rabbit liver total RNA, as well as rabbit liver RNA preparations enriched in poly(A)$^+$ RNA by one (A_1^+) or two (A_2^+) rounds of oligo d(T) is also shown in Figure IV-1. The total RNA preparation contained the least amount of detectable GH receptor mRNA. One round of oligo d(T) resulted in a marked enrichment in poly(A)$^+$ RNA, and two rounds in even a further enrichment.

IGF-I mRNA was not detectable in 30 µg of total RNA from mammary tissue after overnight exposure; the 4.7 kb band detected in mammary RNA is probably the result of nonspecific probe interaction with 28S ribosomal RNA (Figure IV-2A). Even

after long exposure (5 d) of the total RNA blot, a 7.4 kb IGF-I transcript was only barely visible (Figure IV-2B). This transcript was detected in 10 μg of total RNA from bovine, rat, and rabbit liver, as well as in 30 μg of total RNA from human placenta after overnight exposure (Figure IV-2A).

Enrichment of RNA preparations with poly(A)$^+$ RNA by oligo d(T) cellulose chromatography resulted in an increased ability to detect IGF-I mRNA in all tissues (Figure IV-2C, D). This enrichment also resulted in a decrease in the amount of 28S ribosomal RNA present in RNA samples; less hybridization to 28S ribosomal RNA is seen in mammary poly(A)$^+$ RNA compared to total RNA preparations. Binding to 28S ribosomal RNA was also evident in bovine liver total RNA and, to a lesser degree, in total RNA preparations from rat and rabbit liver, and human placenta. This binding was also decreased in poly(A)$^+$ RNA preparations from each of these tissues. A smaller IGF-I mRNA transcript (1.2 kb) was detected in mammary poly(A)$^+$ RNA only after long exposure (Figure IV-2D). This transcript was also present in all liver RNA preparations and human placenta RNA.

The results of an evaluation of nonspecific hybridization of the IGF-I riboprobe to ribosomal RNAs in different mammary RNA preparations are shown in Figure IV-3. The IGF-I riboprobe bound to 28S ribosomal RNA in mammary RNA preparations depleted of polyadenylated RNA (A$^-$ RNA). There was little, if any, difference in the degree of IGF-I riboprobe binding to 28S ribosomal RNA between mammary total RNA and A$^-$ RNA. However, there was a marked decrease in binding to 28S ribosomal RNA seen in mammary poly(A)$^+$ RNA.

Several hybridization and washing conditions were imposed in an attempt to eliminate nonspecific hybridization of the IGF-I riboprobe to ribosomal RNA in mammary tissue. Both higher than normal temperature washing (up to 80°C) and RNase treatment after hybridization decreased the degree of 28S ribosomal binding (data not shown). However, both treatments also eliminated the 7.4 kb band, which is normally detectable in mammary RNA. The intensity of the 7.4 kb band, as well as that of the smaller bands were also decreased in rat liver RNA by both treatments.

No hybridization to ribosomal RNA was detected in mammary RNA preparations, even after long exposure (21 d), using a shorter IGF-I riboprobe generated from Avi II linearized DNA (data not shown). However, there was a considerable loss of sensitivity using the shorter riboprobe. As a result, the 7.4 and 1.2 kb IGF-I mRNA bands were not detectable even in mammary poly(A)$^+$ RNA, although they were still easily detected in rat and rabbit liver RNA preparations.

Effect of Growth Hormone on Insulin-like Growth Factor-I mRNA in Mammary Tissue

The effect of GH injection on the relative abundance of the 7.4 kb IGF-I mRNA transcript in mammary tissue is shown in Figure IV-4. The 7.4 kb transcript was detected in all poly(A)$^+$ RNA preparations obtained from mammary tissue of nontreated animals. The transcript was not detectable in any of the mammary RNA preparations obtained from GH-treated cows. This difference in 7.4 kb transcript abundance, between nontreated and GH-treated animals, was also observed when total RNA blots were subjected to long exposures (5 d). There was no consistent effect of GH injection on the relative abundance of the smaller IGF-I mRNA species (data not shown).

Detection and Effect of Growth Hormone on Type I Insulin-like Growth Factor and Epidermal Growth Factor Receptor mRNAs in Mammary Tissue

Four type I IGF receptor mRNA transcripts of 11.3, 6.2, 4.9, and 3.3 kb were detected in RNA isolated from mammary tissue biopsies (Figure IV-5). These four transcripts were also detected in RNA isolated from human placenta. The 11.3 kb band was observed in bovine, rat, and rabbit liver RNA after long exposure (6 d).

After GH injection, there was a decrease in the relative abundance of all type I receptor transcripts in mammary tissue from two animals. An increase in abundance was observed in mammary tissue from the third GH-treated animal; however, by the end of the study period the feed intake of this animal was less than half the expected intake. This animal was therefore considered to be in a nutrient-deprived state. The changes in type I IGF receptor mRNA abundance were also observed on blots with total and poly(A)$^+$ RNA isolated from postmortem mammary tissue (data not shown).

One EGF receptor transcript of 10.5 kb was detected in mammary tissue (Figure IV-6). There was a decrease in the relative abundance of this transcript in mammary RNA from two GH-treated cows; no change in abundance was observed in RNA obtained from the nutrient deficient cow. The 10.5 kb EGF receptor transcript, and a less abundant transcript (5.6 kb), were also detected in RNA preparations from human placenta, and bovine, rat, and rabbit liver (data not shown).

Localization of mRNAs in Mammary Tissue by *In Situ* Hybridization

Optimization of the in situ hybridization procedure. A number of parameters of the *in situ* hybridization procedure were altered in an attempt to increase sensitivity, while maintaining acceptable tissue morphology and a high signal to noise ratio. The combination of limited proteinase K digestion (up to 10 min), inclusion of 10% dextran sulphate in the hybridization buffer, and the use of short riboprobes (150 bases) produced an additive effect, which markedly increased the intensity of the hybridization signal with all riboprobes used. This general effect is illustrated by comparison of autoradiographic films (XAR-5) exposed (36 h) to tissue sections probed with the type I IGF receptor riboprobe under different conditions (Figure IV-7). Tissue sections that were not digested with proteinase K, and then hybridized with long probes (500-1000 bases) and no dextran sulphate exhibited the lowest intensity hybridization signal (Figure IV-7C). Intermediate signals were obtained using various combinations of treatments other than the aforementioned optimal combination. For example, tissue sections that were digested with proteinase K and then hybridized using 10% dextran sulphate and long probes exhibited an intermediate signal intensity (Figure IV-7B).

Localization of type I insulin-like growth factor receptor mRNA in mammary tissue. In mammary tissue from animals not treated with GH the distribution of type I IGF receptor mRNA was qualitatively similar for all animals. Hybridization was primarily localized over alveolar epithelial cells (Figure IV-8A). Less intense hybridization signals were observed over cells located in the stroma region. The distribution of type I IGF receptor mRNA was also qualitatively similar for all GH-treated animals. There was no apparent change in the general distribution pattern of

hybridization as a result of GH treatment. The most intense hybridization signal was again located over the alveolar epithelial cells, whereas stromal cells exhibited comparably less intense signals (Figure IV-8B). However, there was an overall decrease in the intensity of the hybridization signal in mammary tissue from two GH-treated animals. These two animals were the same two in which there was a decrease in the abundance of type I IGF receptor transcripts observed on Northern blots after GH treatment. There was no noticeable change in the intensity of the *in situ* hybridization signal observed in mammary tissue from the third GH-treated animal.

Localization of epidermal growth factor receptor mRNA in mammary tissue. The distribution of the hybridization signal observed in mammary tissue sections hybridized with the EGF receptor riboprobe was qualitatively the same for all animals, regardless of treatment (Figure IV-9). There were also no apparent differences in the overall intensity of the hybridization signal between GH-greated and nontreated animals. The general distribution of the hybridization signal was similar to that observed for type I IGF receptor mRNA; alveolar epithelial cells exhibited the most intense signals, whereas stromal cells exhibited only a low level of detectable hybridization.

In situ hybridization specificity controls. A number of different controls were evaluated to verify the specificity of the *in situ* hybridization reaction with the type I IGF receptor antisense riboprobe. Figure IV-10 shows the results of several specificity controls in comparison with a hybridization (Figure IV-10A) using an alkaline hydrolyzed type I IGF receptor antisense riboprobe.

Preincubation of tissue sections with excess unlabeled type I IGF receptor antisense riboprobe noticably decreased the specific hybridization signal (Figure IV-10B). Hybridization of tissue sections with type I IGF receptor sense riboprobes resulted in no specific signal (Figure IV-10C). No specific signal was observed when tissue sections were digested with RNase before hybridization (Figure IV-10D). Co-hybridization of tissue sections with labeled and excess unlabeled type I IGF receptor antisense riboprobes also abolished the specific hybridization signal (data not shown). When type I IGF receptor mRNA hybridization was conducted at higher temperatures

than normal (up to 70°C), there was a progressive loss of specific hybridization of signal as the temperature was increased (data not shown). No positive chemographic effects were observed when tissue sections were hybridized with buffer devoid of radiolabeled probe; no negative chemographic effects were observed in NTB-2 coated sections fogged by brief exposure to light (data not shown). The collective results of these controls establish the specificity of the type I IGF receptor mRNA *in situ* hybridization reaction.

Hybridization of tissue sections with IGF-I sense riboprobes resulted in no specific hybridization signal above background (data not shown). However, preincubation of tissue sections with excess unlabeled IGF-I antisense riboprobes, before hybridization with labeled IGF-I antisense riboprobe, did not result in a noticable decrease in the hybridization signal (data not shown). Co-hybridization with labeled and excess unlabeled IGF-I antisense riboprobes also did not decrease the hybridization signal. Similarly, pretreatment of tissue sections with RNase did not abolish the signal in subsequent hybridization. The IGF-I riboprobe used to hybridize tissue sections pretreated with RNase was found to be intact; after incubation on RNase-treated sections, the quality of the riboprobe was examined by gel electrophoresis/autoradiography. Increasing the hybridization temperature (up to 70°C) had little noticable effect on the intensity of the hybridization signal.

The results of the controls to test the specificity of the EFG receptor riboprobe hybridization reaction were qualitatively similar to those observed for type I IGF receptor riboprobe (data not shown). Thus, the results establish the specificity of the EGF receptor riboprobe reaction.

DISCUSSION

Changes in mammary cell physiology are induced when lactating dairy cows are injected with GH. Although the exact nature of these changes is still unknown, they undoubtedly involve stimulation of alveolar epithelial cell division, biosynthesis of milk components, or both of these events. The mechanism by which GH induces these changes in mammary cell physiology is also unknown. The use of RNA analysis methodology has enabled us to demonstrate that GH treatment induces alterations in

the abundance of at least three different mRNAs, whose protein products have been implicated in the regulation of mammary processes.

Isolation and Analysis of Mammary RNA

In order to analyze mRNA in lactating bovine mammary tissue it was first necessary to develop efficient and reproducible RNA analysis procedures. The GIT/CsCl RNA isolation procedure was adopted as the method of choice because its use resulted in reproducible yeilds of RNA from tissue. Addition of ethidium bromide to RNA samples before gel electrophoresis facilitated quantitative comparisons of mRNA abundance between individual samples and obviated the need to run two gels for a single hybridization. Although it has been suggested that this treatment results in a decrease in the efficiency of RNA transfer and hybridization (72, 87), at the concentration used in our protocol neither of these effects were observed. Quantitative and reproducible isolation of poly(A)$^+$ RNA from total RNA using oligo d(T) cellulose chromatography was achieved only through the use of a LiCl buffer system.

The adoption of riboprobes in Northern and *in situ* hybridization procedures contributed much to the overall improvement of each. In preliminary experiments, no IGF-I transcripts of any size were detected on total or poly(A)$^+$ RNA Northern blots probed with random-primer labeled IGF-I cDNA probes of high specific activity (data not shown). Although the probes revealed the presence of IGF-I transcripts in other tissues, such as rat liver, the intensity of the hybridization signal was much lower than that observed in subsequent, comparable blots using IGF-I riboprobes. Detailed discussions describing the advantages of using riboprobes and the chemistry of riboprobe reactions have been presented (48, 51). The sensitivity of the overall Northern hybridization procedure is clearly illustrated by its ability to detect the presence of the 7.4 kb IGF-I mRNA in mammary tissue. To the best of our knowledge, this is the first time that the presence of this low abundance IGF-I transcript has been demonstrated in mammary tissue from any species. Several other studies, which have evaluated IGF-I mRNA in mammary tissue or cells, have not detected this transcript (23, 36, 86).

The combination of limited tissue digestion with proteinase K, inclusion of dextran sulphate in the hybridization solution, and the use of short riboprobes resulted in high intensity hybridization signals in *in situ* hybridization experiments. A low level of background was also maintained with this combination of procedures. Post-hybridization RNase digestion of nonspecifically bound riboprobe was essential to achieving a low level of background. Precise attention to detail in the procedure of liquid emulsion autoradiography was required to prevent artifactual appearance of silver grains.

Insulin-like Growth Factor-I mRNA in Mammary Tissue

Analysis of IGF-I mRNA in bovine mammary tissue led to several important findings. In direct comparison with tissues known to contain IGF-I mRNA, it was established that bovine mammary tissue also contains IGF-I mRNA. However, it was also shown that there is a high level of nonspecific hybridization of the IGF-I probe to 28S ribosomal RNA. Nevertheless, this transcript has been reported as an IGF-I mRNA in a large number of other tissues (12, 33, 37, 45, 54, 57, 59, 86). The transcript has been dectected using IGF-I cDNA probes labeled by various techniques, labeled oligonucleotides, and riboprobes. The abundance of this transcript has also been reported to increase or decrease, depending on hormonal status (37, 57, 60, 70).

The 7.4 kb IGF-I transcript was barely detected in total RNA from bovine mammary tissue, even after long autoradiographic exposure. However, it was readily detected in RNA preparations enriched in poly(A)$^+$ RNA. We also detected two smaller IGF-I transcripts of 1.2 and .5 kb in RNA from mammary tissue. These IGF-I transcripts have also been observed in numerous other tissues from several species, and presumably represent more mature, or nearer fully processed IGF-I transcripts. The transcript of 1.2 kb was actually observed as a range of transcripts from about .8 to 1.4 kb. It has been proposed that these slightly different sized transcripts are the result of variation in the use of different polyadeylation sites on the IGF-I gene (53, 80). Considering that the 7.4 and 1.2 kb transcripts were enriched in poly(A)$^+$ RNA, whereas the 4.7 and .5 kb transcripts were depleted or nonexistent in poly(A)$^+$ RNA, the

authenticity of the .5 kb transcript is also questionable. However, since poly(A)$^+$ tails are continuously shortened during the life of an mRNA molecule (9, 68), it is possible that the .5 kb transcript has a poly(A)$^+$ tail that is too short for effective removal by oligo d(T) chromatography.

The 7.4 kb IGF-I transcript could not be detected in mammary tissue from GH-treated animals, even after long exposure of poly(A)$^+$ blots. This decrease in abundance of the 7.4 kb transcript after GH injection was somewhat surprising; GH treatment generally either increases or does not affect the abundance of IGF-I mRNA in other tissues (53, 55). However, it has recently been demonstrated that GH initially stimulates an increase in the abundance of IGF-I mRNA in cultured hepatocytes, but six hours later there is a decrease to a level less than before GH addition (41). After 24 h of exposure to GH, the mRNA reaccumulates to a level equal to or greater than that observed before GH addition. Immunoreactive IGF-I also accumulated in the hepatocyte culture medium over the 24 h period. Whether the decrease in abundance in IGF-I mRNA in mammary tissue from GH-treated cows also reflects a general increase in tissue IGF-I synthesis is unknown. Nevertheless, lower tissue responsiveness to GH stimulation of IGF-I mRNA accumulation may be due to GH receptor down regulation, induced by the elevated blood GH concentration during GH treatment.

An alternative explanation is that there was a GH-independent decrease in IGF-I mRNA abundance to simply decrease mammary IGF-I synthesis. If an IGF-I autocrine or paracrine mechanism exists to regulate mammary physiology, then this type of response could serve a protective function against overstimulation by IGF-I derived from GH-stimulated IGF-I synthesis in nonmammary tissues. Further research will be required to determine the biological significance of the observed GH effect on the 7.4 kb IGF-I transcript in mammary tissue.

Type I Insulin-like Growth Factor Receptor mRNA in Mammary Tissue

The predominant type I IGF receptor mRNA species in mammary tissue was 11.2 kb. Smaller, less abundant transcripts of 6.2, 4.5, and 3.2 kb were also detected

in mammary tissue. These same transcripts were also observed in RNA isolated from human placenta. The two largest type I IGF receptor transcripts have been detected in human placenta RNA in a previous study (89). An 11.0 kb type I IGF receptor transcript has also recently been detected in rat liver RNA (92).

All type I IGF receptor transcripts were observed in mammary tissue from both nontreated and GH-treated animals. However, there was a marked decrease in the abundance of all receptor transcripts in mammary tissue from two of three GH-treated animals. The abundance of the transcripts actually increased in mammary tissue from the third GH-treated animal. This animal was considered to be in a nutrient-deprived state because her feed intake dropped significantly during the experiment. Considering the well established relationship between IGF-I synthesis and nutritional factors (73, 90), the response observed in this animal may reflect the high priority afforded to mammary tissue function during lactation. If the increase in type I IGF receptor transcript abundance is permissive to receptor synthesis and recruitment to the cell surface, then it may be part of a mammary specific strategy, initiated to maintain maximum functional capacity during periods of nutrient deprivation.

The decrease in type I IGF receptor transcript abundance observed during GH treatment may reflect a receptor regulation process initiated by receptor down regulation. Knowledge of type I IGF receptor biology supports this interpretation. Because this receptor is not stored in an intracellular pool or recycled after internalization (74, 93), it is reasonable to expect that its concentration on the cell surface is regulated through changes in receptor gene transcription, mRNA turnover or stability, or translation rate. Therefore, the decrease in receptor transcript abundance is probably a mechanism to decrease the cell's responsiveness to an increased extracellular concentration of IGF-I, induced by GH injection. This contention is further supported by the finding that type I IGF receptors are down regulated in conjunction with IGF-I action in other cell types (15, 16).

Mammary expression of the type I IGF receptor gene was predominantly localized in alveolar epithelial cells. The reason for the difference in relative abundance

of this receptor mRNA between these cells and stromal cells is unclear. Nevertheless, because lymphocytes and fibroblasts possess type I IGF receptors in all other tissues examined (15, 69, 91), it is likely that in mammary stroma they also possess these receptors. That the alveolar epithelial cells are the prime mammary target for IGF-I action during GH treatment, however, is supported by the collective results of the *in situ* and Northern hybridization analyses.

Epidermal Growth Factor Receptor mRNA in Mammary Tissue

The presence of EGF receptor mRNA (10.5 kb) in bovine mammary tissue was expected because mammary tissue from other species has been shown to possess EGF receptors (11, 62, 85). On the other hand, the GH-induced decrease in EGF receptor mRNA abundance was surprising because, until now, EGF had not been implicated in the lactation response to GH. The simplest interpretation of this change is that alveolar epithelial cells become less responsive to the actions of EGF during GH treatment. This interpretation is consistent with the finding that changes in EGF receptor protein synthesis are due, at least in part, to changes in the abundance of its mRNA (18, 19). The decrease in mammary EGF receptor mRNA abundance may therefore be related to a receptor down regulation process induced by EGF action on mammary cells. It has been demonstrated that EGF receptors are down regulated in response to EGF in several different cell types (11, 52, 56).

A mitogenic action of EGF on mammary cells may induce the release of EGF inhibitory influences, by initiating the process of receptor down regulation. In general terms, this series of EGF actions is consistent with the response to GH injections because milk production increases are not realized until several days after initiation of daily GH injections. Thus, it is conceivable that early proliferative events induced by EGF are followed by differentiative events, induced by both the release of EGF inhibitory influences and by stimulatory influences.

Although this explanation of a role for EGF in GH-stimulated milk production seems plausible, recent information about the regulation of EGF receptors in other cell types suggests that the situation is much more complex. EGF-directed EGF receptor

mRNA accumulation and EGF receptor protein synthesis have recently been demonstrated in hepatic epithelial cells (19). Furthermore, EGF stimulates EGF receptor synthesis in human breast cancer cells (49). This response to EGF is believed to serve a homeostatic function to restore cell surface receptors following receptor down regulation induced by EGF binding. The recent finding of EGF-receptor complex recycling reveals further complexity in the regulation of EGF receptors (82). EGF-directed EGF receptor mRNA accumulation and receptor protein synthesis therefore comprise only part of the EGF response system possessed by a cell. Moreover, the importance and role of these processes in modulating responsiveness to EGF probably varies according to the context of particular EGF actions.

Among other hormones, GH has also been implicated in the regulation of EGF receptor biology. A recent study showed that an experimentally induced pulsatile plasma GH pattern effectively increased hepatic EGF binding and EGF receptor mRNA abundance, whereas continuous GH infusion had little or no effect on either of these parameters (21). In contrast, available evidence does not support the idea that the pattern of plasma GH is important in the regulation of mammary EGF receptors. The plasma GH pattern does not influence the magnitude of GH-induced milk production increases, rather the absolute plasma GH concentration is important in this regard (58). Furthermore, the change in EGF receptor mRNA abundance demonstrated in the present study was induced by single daily GH injections. In view of these apparent differences, a detailed evaluation of the effect of GH on mammary EGF receptors is warranted.

In situ hybridization analysis revealed that the EGF receptor gene is primarily expressed in the alveolar epithelial cells of lactating mammary tissue. The lower hybridization signal observed over cells located in the stromal region suggests that during lactation these cells express the EGF receptor gene at a lower rate, or that EGF receptor mRNA is less stable in these cells. If the abundance of EGF receptor mRNA is proportional to the cell surface complement of EGF receptors, then this finding

suggests that epithelial cells have a relatively higher responsiveness to extracellular EGF.

Mechanism of Growth Hormone Action: Regulation of Mammary Gene Expression

The results of this study of mammary gene expression can be used to propose a mechanism of GH action based on experimental observations. Changes in mammary alveolar epithelial cell physiology induced by GH injection involve either the stimulation of proliferative or functional processes, or some combination of both. Although it was not an objective of this study to determine which of these processes is actually stimulated by GH, data from the study suggest that at least two growth factors are involved in regulating the induction process. This information, together with the knowledge that both growth factors exert potent mitogenic effects on mammary tissue, provides a basis to speculate that at least proliferative processes are stimulated in mammary tissue as a result of GH injection.

It is unlikely that the GH-induced changes in mammary cell physiology are related to mitogenic actions alone. Clearly, the multifunctional nature of both IGF-I and EGF, observed in studies with cultured mammary tissue preparations, should not be ignored. Some or all of the different actions of IGF-I and EGF may contribute to the alterations in mammary cell physiology that ultimately lead to increased milk production. Furthermore, there is also evidence that interactions between these two growth factors operate to regulate mammary physiology. Most notably, they exhibit synergistic activity in stimulating the proliferation of mammary epithelial cells (38, 50, 77, 78). This synergism is further enhanced by the presence of as yet unidentified serum factors. Thus, the nature of the action of each of these growth factors not only depends on the presence of the other, but also on the context set by other substances.

The results of the present study suggest that the actions of IGF-I, EGF, and their homologous receptors are each involved in the changes in mammary epithelial cell physiology induced by GH injection. A direct action of GH can not be excluded because these cells express the GH receptor gene and therefore likely possess functional GH

receptors. This combination of multifunctional signaling molecules is probably not the complete set of regulatory signals that initiate, let alone regulate and maintain the mammary response to GH injection. These signaling molecules represent only part of a larger, more complex combination of regulatory signals that are integrated by alveolar epithelial cells during GH treatment. Identification of the full complement of signals and the molecular mechanisms for their integration remain perhaps the most intriguing challenges in this research area.

Figure IV-1. Isolation of polyadenylated RNA from rat (left panel) and rabbit (right panel) liver RNA using oligo d(T) cellulose chromatography and a LiCl buffer system. RNA was recovered from eluate after total RNA was passed through the column one (E_1), two (E_2), or three (E_3) times. RNA was also recovered from the wash eluate (W). Eluted poly(A)$^+$ RNA was recovered after one (A_1^+) and two (A_2^+) rounds of chromatography. Total RNA (T) is also shown. Each lane contains 10 μg of RNA. Membrane was probed with a GH receptor antisense riboprobe. Band sizes are indicated in kilobases.

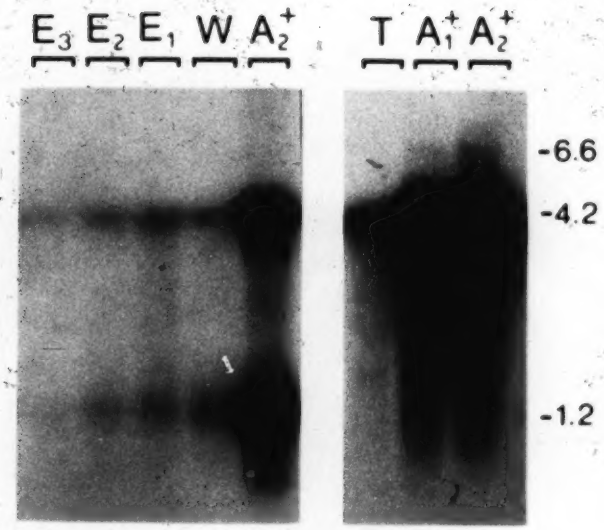

E3 E2 E1 W A2+
T A1+ A2+
-6.6
-4.2
-1.2

Figure IV-2. Detection of IGF-I mRNA in mammary tissue. Total (A, B) and poly(A)$^+$ (C, D) RNA blots, hybridized with the IGF-I antisense riboprobe, were exposed to x-ray film for 20 h (A, C) and 5 d (B, D). Total RNA from mammary tissue (M, 30 μg), human placenta (P, 30 μg), and bovine (B, 10 μg), rat (R, 10 μg), and rabbit (Rb, 10 μg) liver are shown. Poly(A)$^+$ RNA blots contained 20 μg (M, P) or 8 μg (B, R, Rb) RNA. Band sizes are indicated in kilobases.

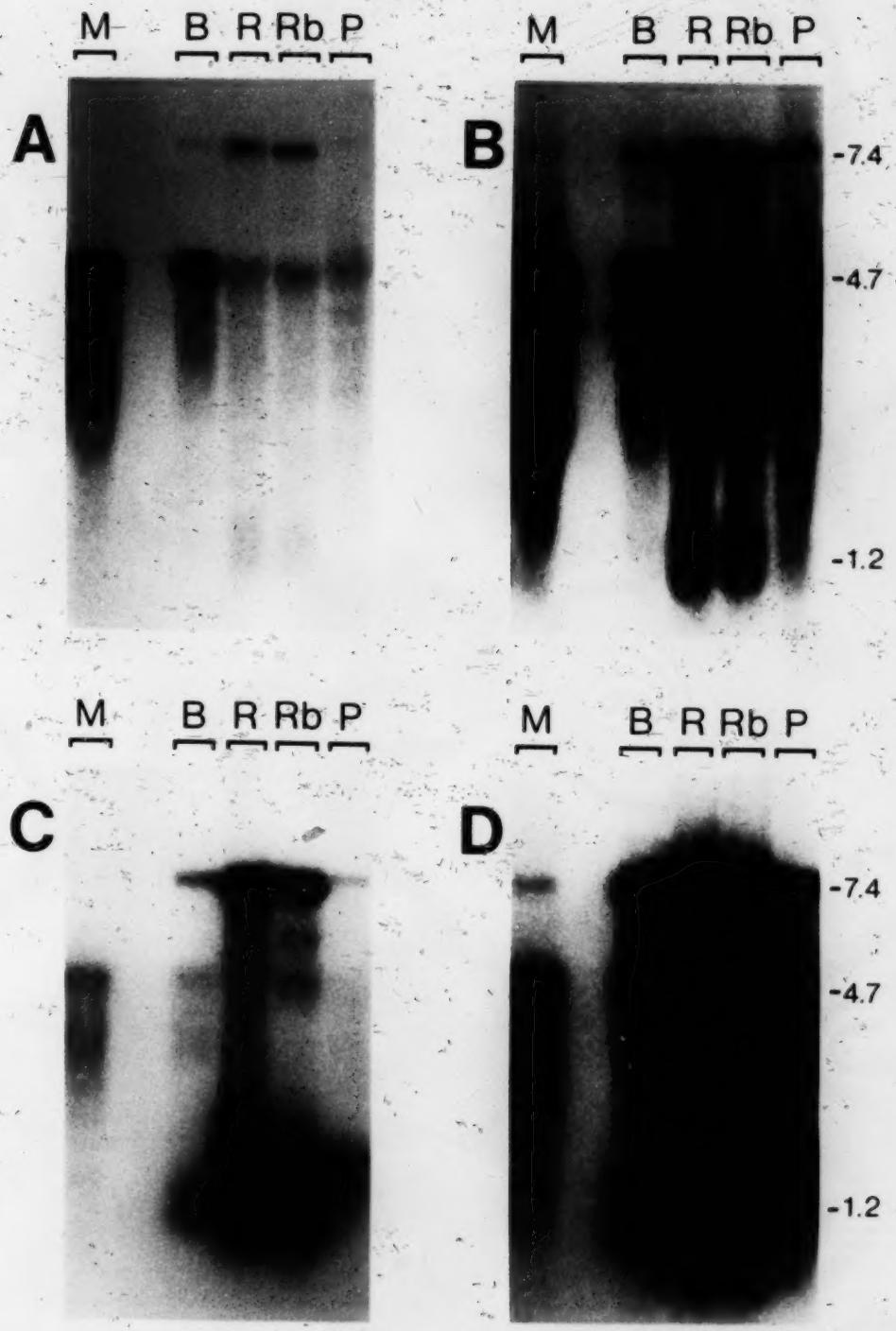

M
B
R
Rb
P
A
B
C
D
-7.4
-4.7
-1.2

Figure IV-3. Nonspecific hybridization of IGF-I antisense riboprobe to mammary 28S ribosomal RNA. Hybridization is observed with total (T) and polyadenylated (A^+) RNA, and RNA depleted of polyadenylated RNA (A^-). There are 30 μg and 20 μg RNA in each lane on total and polyadenylated RNA blots respectively.

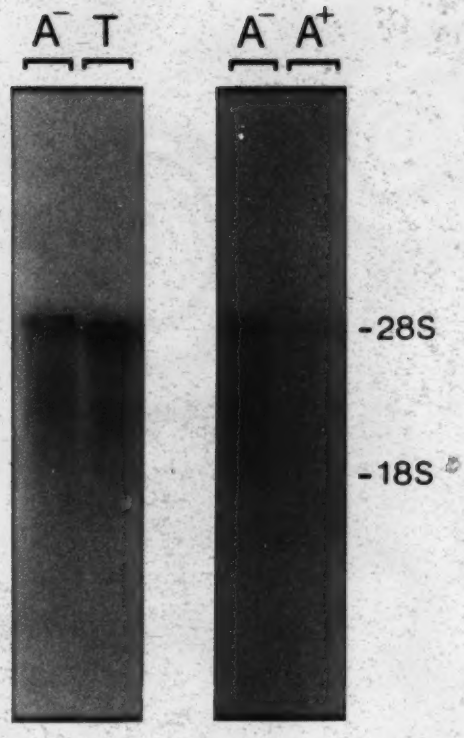

A⁻ T
A⁻ A⁺
-28S
-18S

Figure IV-4. Effect of GH treatment on IGF-I mRNA in mammary tissue. Poly(A)$^+$ RNA (20 μg) from mammary tissue of GH-treated (GH) and normal (N) cows are shown. Band size is indicated in kilobases.

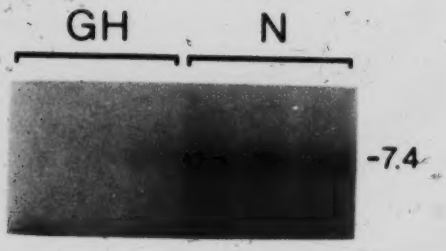

GH
N
-7.4

Figure IV-5. The effect of GH treatment on type I IGF receptor mRNAs in mammary tissue. Total RNA (12 μg) from mammary tissue biopsies of GH-treated (GH) and normal (N) cows are shown. Total RNA from bovine liver (B, 4 μg), rat liver (R, 4 μg), rabbit liver (Rb, 4 μg), human placenta (P, 12 μg), and mammary RNA depleted of polyadenylated RNA (A, 12 μg) are also shown. Band sizes are indicated in kilobases.

A
GH
N
B
R
Rb
P
-11.3
-6.2
-4.9
-3.3

Figure IV-6. Effect of GH treatment on EGF receptor mRNA in mammary tissue. Total RNA (30 μg) from mammary tissue of GH-treated (GH) and normal (N) cows are shown. Mammary RNA depleted of polyadenylated RNA (A, 30 μg) is also shown. Band sizes are indicated in kilobases.

A
GH
N
-10.5

Figure IV-7. Comparison of *in situ* hybridization signal intensity obtained using mammary tissue under different hybridization conditions. The most intense signal (A) was obtained using proteinase K digestion, dextran sulphate, and short (150 bp) antisense riboprobes. Intermediate intensity (B) was obtained using long (1000 bp) antisense riboprobes. The least intense signal (C) was obtained using long probes without dextran sulphate on sections not treated with proteinase K. Sections were hybridized with type I IGF receptor antisense riboprobes. Images are recorded on x-ray film.

A
B
C

Figure IV-8. *In situ* hybridization of radiolabeled type I IGF receptor antisense riboprobe to mammary sections from normal (A) and GH-treated (B) cows. The right panels are darkfield images of the same section location shown in brightfield illumination in the left panels. Examples of alveolar epithelial cells (e) and stroma (s) are indicated. Bars represent 10 μm.

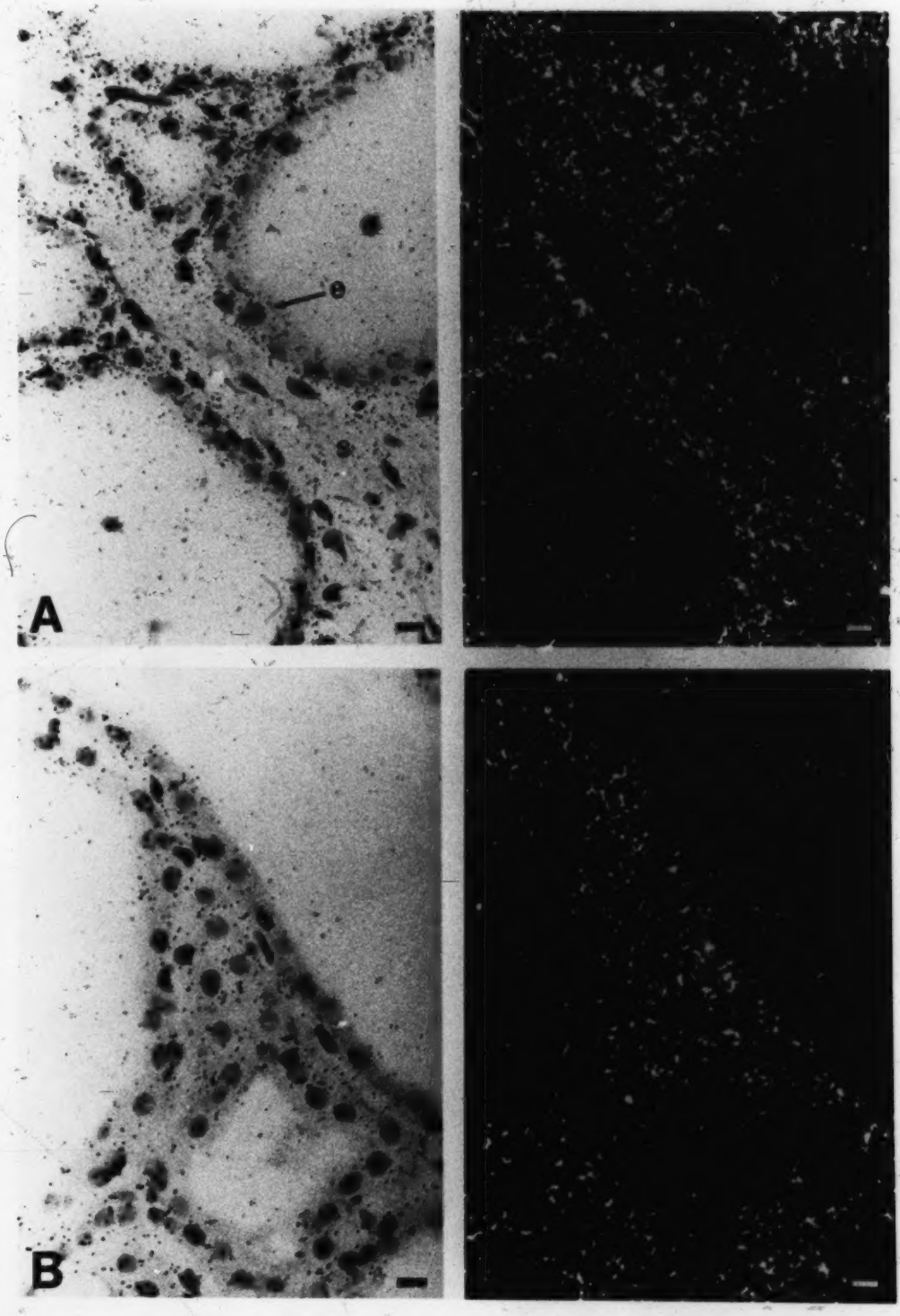

A
B

Figure IV-9. *In situ* hybridization of radiolabeled EGF receptor antisense riboprobe to sections of normal lactating mammary tissue. The right panel is a darkfield image of the same section location shown in brightfield illumination in the left panel. Examples of alveolar epithelial cells (e) and stroma (s) are indicated. Bars represent 10μm.

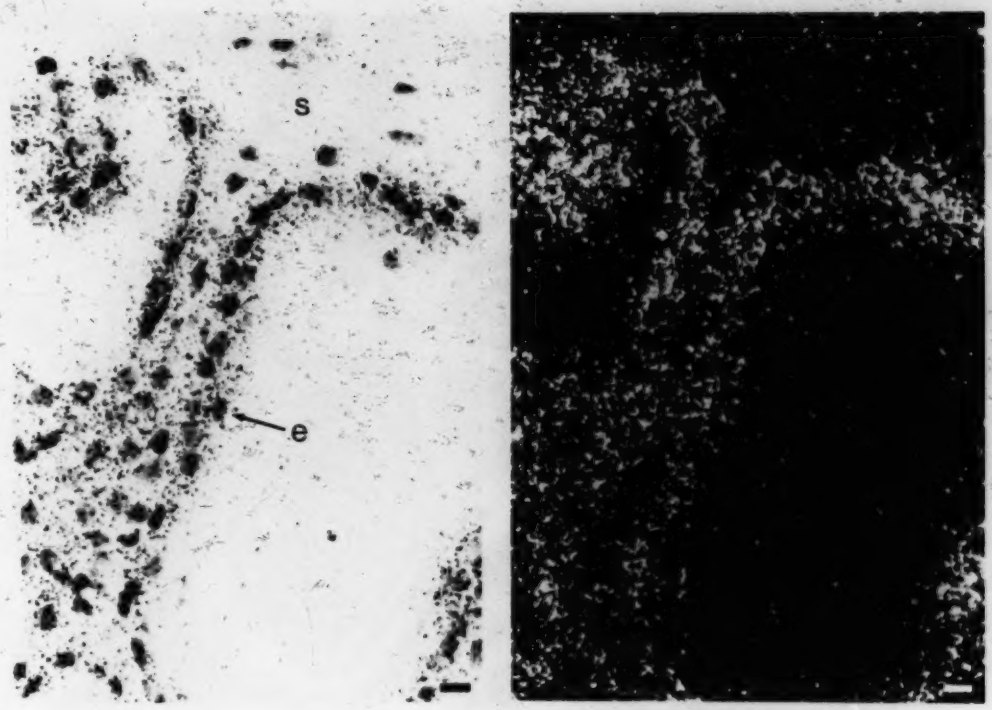
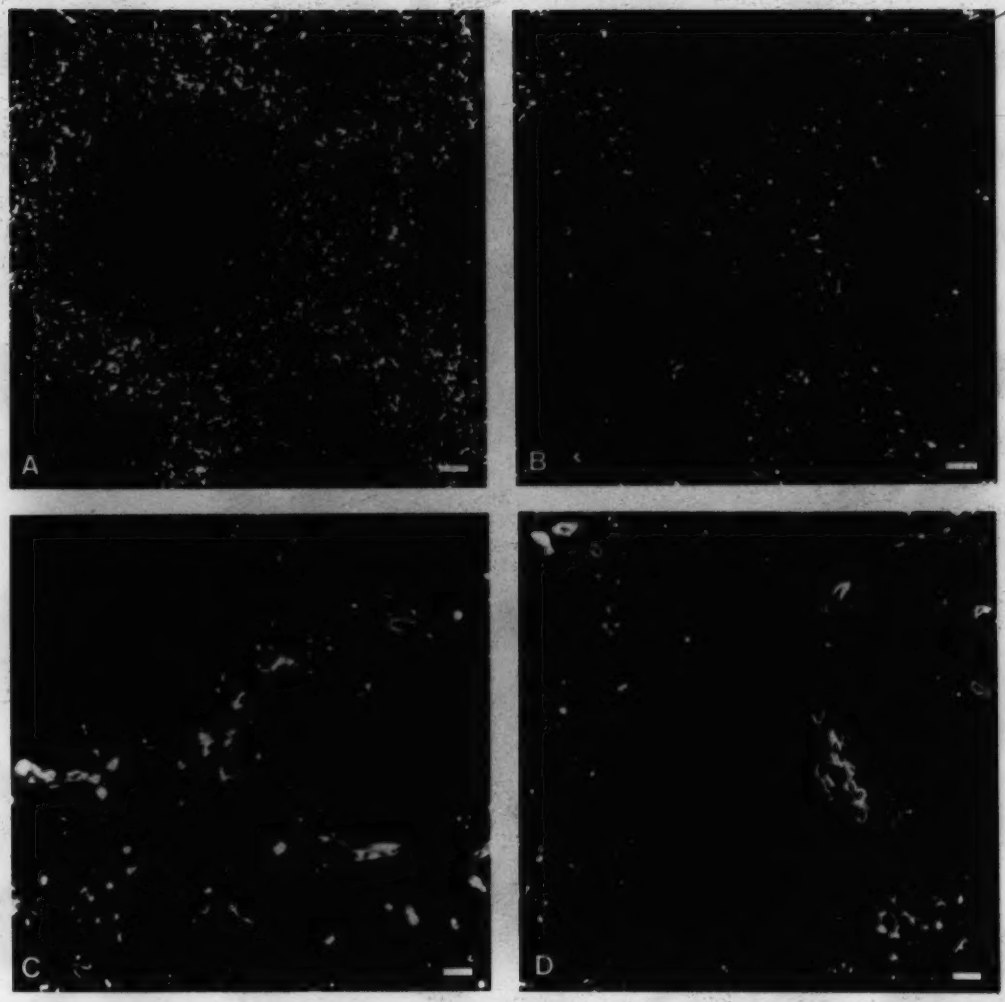

s
e

Figure IV-10. *In situ* hybridization specificity controls. All mammary tissue sections were hybridized with Type I receptor riboprobes. For comparison, a section hybridized with only the antisense riboprobe is shown (A). Specificity controls included preincubation with excess unlabeled antisense RNA (B), hybridization with sense riboprobe (C), and hybridization to sections pretreated with RNase (D). Bars represent 10 μm.


A
B
C
D

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GENERAL DISCUSSION AND CONCLUSIONS

The purpose of this thesis is to provide insight into the role of GH in bovine mammary biology. A survey of the current literature related to this subject revealed that little is known about the function of GH in mammary processes. However, it has been repeatedly demonstrated that GH injection of lactating dairy cows results in a marked increase in milk production (53). This reponse clearly indicates that the action of GH is important in the regulation of mammary epithelial cell physiology, at least during lactation. The nature of the GH-related changes in epithelial cells and the mechanisms that induce these changes are the major issues addressed by this thesis.

Our general lack of knowledge of mammary biology limits progress in the dairy industry. This lack of knowledge is also the basic reason we are unable to stop breast cancer, which effects one in ten North American women and contributes directly to the death of one out of four women with the disease (57). Not surprisingly, most of the progress made so far in understanding the physiology of mammary epithelial cells has in fact come from breast cancer research. There is a need to know details of normal mammary processes to understand alterations which lead to disease.

The mammary epithelial cell is only one of more than 120 different epithelial cell types in an adult animal (3). Besides epithelial cells there are about another 100 different major cell types. This diversity in phenotypes alone reveals the complexity of the regulatory system that must exist to control the normal growth and function of cells. However, it is apparent that the underlying mechanism of this regulatory system is based on the use of a large number of signaling molecules with a collective informational content sufficient to control the physiology of all cell types. The identification of the different combinations of signaling molecules relevant to the regulation of mammary epithelial cell biology, at each different stage of mammary development, continues to be a major challenge for dairy and breast cancer research. As a part of this larger challenge, there is now a new challenge to identify the combination of signaling molecules which induce the mammary changes associated with GH injection. This thesis represents some of the first research to meet this new

challenge. Knowledge of how GH interacts with other signaling molecules in the control of mammary cell physiology will increase our overall understanding of mammary biology.

Available information about the role of GH in mammary processes often conflicts and is at best confusing. This is mostly due to inconsistencies of *in vitro* systems and contamination in hormone preparations, as well as the general difficulty of maintaining cultured mammary cells and explants (17, 37, 47, 48, 62, 64). Recent developments such as serum-free culture conditions and more information about substratum requirements have resulted in more useful *in vitro* mammary systems (1, 6, 46, 60, 62, 68). Nevertheless, results from *in vitro* experiments should still be interpreted with caution in view of the complexity in regulation of cell physiology and the general inadequacy of *in vitro* systems in mimicking the *in vivo* environment. The critical nature of proper spatial organization of mammary epithelial cells and remaining uncertainties about substratum interactions are particularly important in this regard.

Production Effects of Growth Hormone Treatment: Clues to the Role of Growth Hormone in Mammary Biology

The production and general health effects of GH treatment of dairy cattle are well established (10, 53). The wealth of information contributed by production studies is most valuable in terms of the development of strategies for GH application in the dairy industry. Nevertheless, some information from these studies provides clues to the nature of mammary changes induced by GH injection.

The general lack of an effect of different patterns of GH administration on the milk production response may be relevant in terms of the regulation of mammary receptor biology (48). It has been demonstrated that GH down regulates type I IGF receptors *in vitro* (72). By contrast, EGF receptors are up regulated by GH, although this is apparently dependent upon the nature of the GH plasma pattern (27). It has also been shown that GH has the ability to both down regulate and up regulate its own receptors (56). If any of these receptor processes occur in mammary tissue during GH treatment, then not only could homologous ligand actions be altered, but it is also

possible that heterologous ligand actions could be altered through interactions between receptors. However, because the milk production response to GH injection does not change under different GH administration patterns, any receptor events that do occur must be conducive to processes that increase milk production. The question of whether changes in GH concentration in the mammary epithelial cell's environment results in receptor regulatory events awaits further study.

The effect of GH injection on milk composition provides another clue to the nature of at least some of the mammary changes. It is noteworthy, however, that this effect has only recently been revealed through studies that have evaluated the efficacy of sustained-release GH preparations (9); normally, daily GH injection does not result in changes in milk composition (8). The administration of sustained-release GH preparations, once every two weeks, presumably results in the delivery of a diminishing quantity of GH toward the end of the two week injection interval. This assumption is based on the observed cyclic pattern in milk yield. A progressive increase in milk yield occurs up to day seven of the injection interval and then there is a progressive decrease toward the end of the injection interval. Changes in milk protein, and to a lesser degree milk fat, also exhibit cyclic patterns within injection intervals. However, these changes do not consistently parallel the cyclic change in milk yield. On the other hand, changes in milk lactose content parallel milk yield changes. This differential effect of GH on milk constituents probably reflects subtle differences in temporal occurance of the biochemical changes in epithelial cells required for increased milk component synthesis.

In view of this differential response in milk composition it is tempting to speculate that the GH-induced mammary changes are functional rather than proliferative in nature. However, new cells from a proliferative event would also exhibit temporally discordant functional changes during differentiation. Thus, both functional and proliferative mammary changes could be responsible for the noncoordinate effect of GH on the synthesis of different milk constituents.

Hypothesis of Growth Hormone Action

It is apparent that there is a wide acceptance of a mechanism of GH action in lactating dairy cows based on nutrient partitioning to the mammary gland (28, 36, 53, 60). It is held that the primary action of GH is to orchestrate metabolic processes such that more nutrients are partitioned to the mammary gland to support increased milk synthesis. Although such metabolic effects are likely an important part of the response to GH, alone they do not adequately explain the mechanism of GH action. Clearly, changes must also occur in the mammary gland in order for milk production to increase. This prerequisite for increased milk synthesis leads to the inevitable question, of whether changes in the mammary gland are a consequence of metabolic changes in nonmammary tissues or metabolic changes are a consequence of mammary changes.

Understanding the regulatory mechanisms that induce metabolic changes in nonmammary tissues during GH injection would require an almost complete understanding of the regulation of intermediary metabolism during lactation. The hypothesis proposed in this thesis, of the mechanism of GH action, accounts for this fundamental requirement of regulatory complexity. It is proposed that the metabolic changes, which occur in nonmammary tissues during GH treatment, are initiated by signals derived from mammary tissue. Moreover, the signal complement is most similar to that of the signals which operate during early lactation. As a corollary, the mammary-derived early lactation regulatory signals induce the same metabolic changes in nonmammary tissues that are induced during GH treatment. The finding that the response of free fatty acid release to epinephrine challenge is enhanced in early lactation and also during GH injection illustrates the common metabolic responsiveness of adipose tissue in both early lactation and GH-stimulated lactation (49). Future research will probably delineate additional similarities in the metabolism of other nonmammary tissues. There is little doubt that the induction of metabolic changes in nonmammary tissues, in both instances, is a complex process involving a large number of signaling molecules.

The other component of the hypothesis of this thesis is that the primary response to GH injection involves actions on the mammary gland. As a result of this response, the alterations in mammary physiology ultimately induce metabolic changes in nonmammary tissues. According to the hypothesis, in the primary response, GH must directly or indirectly act on the mammary gland. Both of these possibilities were investigated in the research conducted as a part of this thesis.

Serum Insulin-like Growth Factor-I Induction by Growth Hormone

Based on the lack of supporting evidence for a direct action of GH on mammary tissue, the possibility of mediator involvement was addressed in the first series of experiments. The mediator chosen for study was insulin-like growth factor-I (IGF-I). The rationale for this choice was primarily based on the finding that the effect of GH on tibial cartilage growth in rats was actually induced by locally synthesized IGF-I (59). The results of other studies also supported a role for IGF-I in inducing certain physiological effects attributed to GH (35, 51, 52). Furthermore, there was at least a basis to speculate that cows treated with GH would have an increased blood IGF-I concentration. This basis was provided by studies that showed a blood IGF-I induction response to GH injection in a number of different species (21, 70). Type I IGF receptors had also been identified on human breast cancer cells and IGF-I stimulated DNA synthesis in these cells (32). There was also evidence to suggest that IGF-I regulated carrier-mediated glucose transport in mammary explants from pregnant mice (55).

The first experiment of this thesis addressed the question of whether GH injection would increase blood IGF-I concentration in lactating dairy cows. In order to conduct these experiments several different IGF-I radioimmunoassay procedures were critically evaluated. The most reproducible protocol was chosen, and then modified and validated for use with bovine serum. The dose of recombinant bGH used in the first experiment was the same dose that had been consistently demonstrated to induce a milk production response when administered as daily injections (10, 29). The major finding of this experiment was that a single bGH injection induces about a two-fold

increase in serum IGF-I concentration by 18 hours after injection. This serum IGF-I induction response to GH injection was confirmed in a second experiment involving three consecutive daily bGH injections. The finding that GH injection markedly induced serum IGF-I in lactating dairy cows provided the first direct evidence in support of the supposition that IGF-I was involved in the lactation response to GH.

Insulin-like Growth Factor-I and its mRNA in Mammary Tissue

The first direct evidence to implicate IGF-I specifically in the mammary response to GH was obtained through immunocytochemical experiments with mammary tissue. It was shown that the IGF-I protein was present in lactating mammary tissue, and that it accumulated in epithelial cells during GH treatment. Thus, it was established that IGF-I was either internalized or synthesized by epithelial cells during GH treatment. The finding of specific IGF-I receptors on bovine mammary epithelial cells established that these cells have the capacity for biological responsiveness to IGF-I (14). Furthermore, this finding supported the view that IGF-I accumulation in epithelial cells during GH treatment was most likely the result of IGF-I internalization by homologous receptor-mediated endocytosis. The possibility that epithelial cells synthesize IGF-I could not be excluded, however.

RNA analysis methodology was used to determine if IGF-I was in fact synthesized locally in the mammary gland. Both the presence of IGF-I mRNA and an abundance change in response to GH injection were established in mammary tissue from lactating dairy cows. However, because IGF-I mRNA proved to be such a low abundance mRNA in mammary tissue, it was necessary to develop a very sensitive Northern hybridization procedure. This was accomplished through modification of existing procedures in conjunction with integration of recently developed techniques.

An important methodogical consideration became apparent during the development of the RNA analysis procedure; there is a stringent requirement for controls in Northern hybridization experiments. The collective results of our IGF-I mRNA analysis experiments clearly illustrate this point. The results establish that both IGF-I riboprobes and cDNA probes spuriously hybridize to 28S ribosomal RNA.

Moreover, a survey of the literature regarding IGF-I mRNA analyses showed a lack of recognition of this problem. This oversight has led to the erroneous interpretation of data from IGF-I mRNA analysis experiments. The types of hybridization methodology experiments susceptible to this type of problem include those involving dot blot, Northern, solution, RNase protection, and *in situ* hybridizations. In view of this problem, the results of IGF-I mRNA experiments should be carefully reevaluated and future IGF-I mRNA experiments should include the use of proper controls.

Our finding that IGF-I mRNA was synthesized in mammary tissue was exciting because it supported the concept of local control of mammary physiology. Furthermore, researchers in the breast cancer field also used immunocytochemistry to demonstrate that breast cancer cells contain IGF-I (65). Subsequent experiments showed that several breast cancer cell lines actually secrete IGF-I *in vitro* (23). These findings supported the concept of local mammary growth control and prompted a series of IGF-I-related studies in breast cancer research.

Two recent breast cancer studies reported the presence of IGF-I mRNA in breast cancer cells (31, 38). In both studies, however, a hybridizing band of 4.7 kb is assumed to be IGF-I mRNA without the use of controls. Smaller IGF-I mRNAs are also reported in these studies. It is likely that future experiments using controls will characterize breast cancer cell IGF-I mRNAs in detail.

Recent studies on breast cancer have explored the possibility of inhibiting breast cancer cell growth using a monoclonal antibody that binds to the type I IGF receptor (4, 5). The antibody inhibits breast cancer cell growth in the presence of serum, but does not inhibit basal growth of cells in serum-free culture medium. These findings further support a role for IGF-I in breast cancer cell growth. However, they also suggest that locally synthesized IGF-I is not important in terms of tumor cell autocrine growth control, at least not through a mechanism involving the type I receptor.

Insulin-like Growth Factor-I Gene Expression in Mammary Cells

Since we had identified both IGF-I in bovine mammary epithelial cells and its mRNAs in RNA preparations from mammary tissue, it was of interest to determine

which mammary cells express the IGF-I gene. The only way this could be accomplished was by using the technique of *in situ* hybridization. Although the technique had been only recently introduced, its rapid adoption by researchers in diverse scientific fields led to a wealth of procedural anecdotes and variations (20, 69). Using this information in conjunction with a basic protocol, a sensitive and reproducible *in situ* hybridization protocol using mammary tissue was developed. The application of appropriate controls in this technique also proved to be essential to verify the specificity of hybridization signals. Most notably, and again using the IGF-I riboprobe, it was shown that certain controls can support specificity while at the same time others may question it. Only the IGF-I antisense control resulted in negligible *in situ* hybridization; four other controls did not convincingly support the specificity of the IGF-I *in situ* hybridization reaction. Based on this ambiguity, we have chosen not to present the results of IGF-I *in situ* hybridization experiments. Thus, the question of which cell types in bovine mammary tissue express the IGF-I gene remains unresolved. The lack of an answer to this biological question, however, does not lessen the possibility of local control of mammary physiology by IGF-I.

Growth Hormone Regulation of Insulin-like Growth Factor-I mRNA in Mammary Tissue

Our finding that the abundance of the 7.4 kb IGF-I transcript is markedly decreased in mammary tissue from GH-treated cows was somewhat surprising; GH treatment generally either increases or does not affect the abundance of IGF-I transcripts in other tissues (21). More recently, however, it has been demonstrated that GH initially stimulates an increase in the abundance of IGF-I transcripts in cultured hepatocytes, but six hours later there is a decrease to a level less than before GH addition (41). After 24 hours of exposure to GH, the transcript reaccumulates to a level equal to or greater than that before GH addition. Immunoreactive IGF-I also accumulated in the culture medium over the 24 hour period. Although not direct evidence, this finding supports the idea that the decrease in abundance of IGF-I mRNA in GH-treated mammary tissue is associated with an overall increase in tissue IGF-I

synthesis. It is conceivable that mammary GH receptor down regulation, induced by the elevated blood GH concentration during GH treatment, may result in a transient decrease in responsiveness to GH-stimulated IGF-I mRNA accumulation.

An alternative explanation for the decrease in IGF-I transcript abundance is simply that there was a decrease in IGF-I gene transcription or transcript stability to effectively inhibit mammary IGF-I synthesis. If an IGF-I autocrine or paracrine mammary growth control mechanism exists, then this type of response may reflect protection against overstimulation by IGF-I derived from GH-stimulated IGF-I synthesis in nonmammary tissues.

Type I Insulin-like Growth Factor Receptors in Mammary Tissue

Although specific IGF-I receptors had been identified on human breast cancer cells (32), we decided to attempt to extend this observation to bovine mammary tissue. Using a type I IGF receptor cDNA probe, type I receptor mRNA was barely detectable in poly(A)$^+$ RNA preparations from bovine mammary tissue. Nevertheless, in preliminary experiments we observed that the abundance of this mRNA decreased to an undetectable level in mammary tissue from GH-treated cows. We subcloned the type I IGF receptor cDNA into a plasmid with transcription capability in order to enhance our ability to detect this mRNA. Using riboprobes generated from this subclone it became easy to detect type I receptor mRNA, even in total RNA preparations from mammary tissue. Moreover, the apparent decrease in abundance of this mRNA in mammary tissue from GH-treated cows was confirmed using riboprobes.

This mRNA analysis data, together with the finding of specific IGF-I receptors on microsomes prepared from mammary tissue of pregnant and lactating cows (14), provided a basis to speculate that at least some cell types in bovine mammary tissue were targets for IGF-I action. To delineate which cell types express the type I IGF receptor gene, we again applied *in situ* hybridization methodology. After establishing the specificity of the *in situ* hybridization reaction, we were able to conclude that the receptor gene is predominantly expressed in the alveolar epithelial cells. We also observed a general decrease in the hybridization signal in mammary tissue sections

from GH-treated cows, in agreement with results obtained by Northern hybridization analysis.

Because there is no information connecting mammary type I IGF receptor changes to mammary responses to IGF-I, we can only speculate about the significance of these findings. Assuming that the GH-related decrease in receptor transcript abundance reflected receptor down regulation, then two possibilities arise. First, epithelial cells may have simply reduced their responsiveness to IGF-I in the face of high circulating IGF-I concentration. Secondly, type I receptor down regulation could have occured as a result of receptor-mediated endocytosis. The second response seems more likely because it is generally observed in conjunction with IGF-I action on cells (19, 22). IGF-I-receptor complex internalization is followed by lysosomal degradation of the complex, and there is believed to be negligible recycling of this receptor (58). The absence of a receptor recycling regulatory mechanism suggests that transcription or mRNA turnover regulatory mechanisms may exist. Thus, the simplest and most plausible explanation for the decrease in receptor transcript abundance is that it reflects a receptor regulatory mechanism initiated by IGF-I biological action on epithelial cells. This action may be relevant to the mammary response to GH treatment.

Growth Hormone Receptors in Mammary Tissue

Although it had become widely accepted that there were no GH receptors in the bovine mammary gland, this was difficult to reconcile considering the heterogeneous nature of this tissue. This acceptance was largely based on the results of studies that failed to demonstrate significant [^{125}I] bGH binding to mammary membrane preparations (2, 33, 43, 44). The lack of significant effects of bGH on mammary tissue in culture was also used as evidence to support the notion that the tissue did not possess GH receptors (53).

The cloning of the GH receptor gene provided an opportunity to resolve this question. To facilitate detection and analysis of GH receptor mRNA we subcloned the GH receptor cDNA insert into a transcription plasmid. Using riboprobes generated from the subclone we easily detected GH receptor mRNA in both total and poly(A)$^+$ RNA

preparations from mammary tissue. This finding is important because it reestablishes the concept of direct GH action on mammary physiology.

In situ hybridization analysis revealed that the GH receptor gene was in fact expressed predominantly in alveolar epithelial cells of lactating mammary tissue. This finding may explain the apparent ability of GH to potentiate the maintenance of fat synthesis in lactating bovine mammary explants in culture (30). Similarly, it may explain other reports of subtle or inconsistent effects of GH on casein synthesis and lactose production in bovine mammary tissue explants (34, 63). There is obviously now a need to reevaluate the role of GH in regulating epithelial cell physiology during lactation, and at other stages of mammary development as well.

Epidermal Growth Factor Receptors in Mammary Tissue

Based on the well established importance of EGF in mammary physiology in several species, we decided to investigate the possibility of EGF involvement in the lactation response of cows to GH injection. Furthermore, it was apparent, at least in the mouse, that the complement of mammary EGF receptors was an important determinant of EGF-regulation of mammary physiology (26). In view of this relationship, we conducted Northern and *in situ* hybridization experiments with mammary tissue preparations and an EGF receptor riboprobe, in an attempt to identify and characterize EGF receptor mRNA in lactating bovine mammary tissue. EGF receptor transcripts, of the same sizes as those identified in other tissues, (24, 25, 27, 39) were detected in mammary tissue. Our *in situ* hybridization experiments demonstrated that the EGF receptor gene is expressed predominantly in epithelial cells of lactating tissue.

We also observed a decrease in EGF receptor mRNA abundance in mammary tissue from GH-treated cows. Because EGF has been shown to induce both homologous receptor up and down regulation (16), depending on cell type, it is possible that the change observed in EGF receptor mRNA abundance in mammary tissue could reflect a response to either of these events. In breast cancer cells, however, EGF induces EGF receptor down regulation that is followed by an increase in receptor protein synthesis

(45). This general pattern is also observed in rat liver epithelial cells and KB cells (18, 25). Further, the increase in receptor protein synthesis in these two cell types is at least partly due to enhanced accumulation of EGF receptor mRNA. Whether an EGF-EGF receptor coupling event preceded, and was ultimately responsible for the change in abundance of receptor mRNA observed in mammary tissue is unknown. It is noteworthy that other hormones, including GH have also been implicated in the regulation of EGF receptors in various cell types (16, 27, 42, 71). Clearly, much remains to be learned about the role of EGF and its receptors in bovine mammary epithelial cell physiology.

Recent Research Relevant to the Thesis Hypothesis

Support for a role of IGF-I in mediating the response to GH treatment is provided by two preliminary reports on the effects of IGF-I on bovine mammary tissue explants in culture (11, 12). The reports indicated that IGF-I was a potent mitogen, at least *in vitro*, for bovine mammary tissue from both pregnant and lactating cows. Although one of the reports also indicated that IGF-I stimulated lactose production in lactating acini cultures (11), this was believed to be a consequence of IGF-I-induced cell proliferation (C. Baumrucker, personal communication).

Most recently, the aforementioned preliminary findings have been confirmed and extended by two more complete reports on the *in vitro* effects of IGF-I on bovine mammary tissue (13, 60). It is apparent that IGF-I stimulates epithelial cell growth in mammary tissue from virgin, pregnant, and lactating cows. On the other hand, IGF-I apparently affects neither fatty acid synthesis nor α-lactalbumin secretion in lactating bovine mammary explants (60). It has been suggested that this lack of stimulation could have been due to the accumulation of milk components, which results in a condition referred to as milk stasis (13).

The presence of GH receptors on mammary epithelial cells is consistent with a model of GH-induced IGF-I autocrine action on these cells. However, the results of a recent experiment with human breast cancer cells could be considered evidence against this type of mechanism (31). Neither IGF-I secretion nor IGF-I mRNA abundance were

altered as a result of GH addition to culture breast cancer cells. However, the absence of adequate controls for Northern hybridization in this study resulted in failure to recognize that the IGF-I probe spuriously hybridized to 28S ribosomal RNA. Thus, the IGF-I mRNA response to GH should be reevaluated. On the other hand, it has also been shown that an IGF-I monoclonal antibody can inhibit cell proliferation under serum-free conditions (4, 5). Collectively, the results from these studies support a role for IGF-I in breast cancer cell growth regulation, although the regulation is seemingly GH independent.

Another recent preliminary report indicates that bovine mammary tissue cultured in the presence or absence of GH does not synthesize IGF-I (15). Researchers have also been unable to detect IGF-I mRNA in mammary tissue from pigs (67). By contrast to these seemingly negative findings, another recent preliminary report indicated that ovine mammary tissue does produce IGF-I *in vitro* (73). Future research will undoubtedly resolve the issue of whether mammary tissue synthesizes IGF-I at different stages of mammary development in some or all species. The data from our analyses of IGF-I mRNA certainly suggest that bovine mammary tissue synthesizes IGF-I and that, at least in part, its synthesis is GH dependent. Our finding of GH receptor mRNA in mammary tissue substantiates this conclusion.

The most recent study in this research area involved intra-arterial infusion of IGF-I into the mammary gland of lactating goats (54). The treatment resulted in a 25% increase in milk production from the infused gland. This finding established an *in vivo* role for IGF-I in the regulation of mammary function. Furthermore, it also substantiates the conclusion in this thesis that IGF-I plays a mediator role in GH enhancement of lactation.

Conclusions

In summary, the purpose of this thesis was to gain insight into the molecular and cellular bases of the role of GH in bovine mammary gland biology. The research reported in this thesis used lactating dairy cows injected with GH as an experimental model because it was established that this treatment markedly stimulates milk

production. Clearly, an understanding of the mechanism underlying the response to GH would provide insight into the role of GH in mammary cell biology.

The hypothesis of this thesis is that the mechanism of the response to GH is a primary induction of physiological changes in mammary cells. In turn, these changes ultimately initiate a series of metabolic alterations in nonmammary tissues. Eventually, metabolism is adjusted to completely support the need for additional substrates for increased synthesis of milk components.

The signals from mammary tissue that initiate changes in the metabolism of nonmammary tissues are proposed to be the same signals that initiate similar changes during early lactation, without GH treatment. Considering the obvious complexity of this general regulatory process, it probably involves a large number of signaling molecules. On the other hand, it is plausible that relatively fewer signals could initiate the primary response in mammary tissue. Even the possibility that GH alone initiates the primary response can not be excluded, though it seems unlikely. GH probably induces a set of regulatory molecules with sufficient informational content in the proper context to direct the primary response in the mammary gland.

The primary response to GH injection is either enhanced growth or function of mammary epithelial cells, although a combination of both responses could also explain the increase in milk production. Identification of the regulatory molecules that induce the primary changes in epithelial cell physiology is a key step in understanding the molecular mechanism of GH action. The overall objective of the research conducted for this thesis was to identify regulatory molecules that could induce changes in epithelial cell physiology during GH treatment. Several regulatory molecules including IGF-I, IGF-I mRNA, type I IGF receptor mRNA, GH receptor mRNA, and EGF receptor mRNA were studied in both normal and GH-treated lactating dairy cows. The experiments on these regulatory molecules led to several major findings:

1. GH injection induces a marked increase in serum IGF-I concentration of lactating dairy cows.

2. The IGF-I protein is present in lactating mammary tissue, associated mostly with cells in the stromal region. GH treatment results in an accumulation of IGF-I in alveolar epithelial cells.
3. Mammary alveolar epithelial cells express the GH receptor gene.
4. The IGF-I gene is expressed in lactating mammary tissue. GH treatment results in a decrease in the abundance of the largest detectable IGF-I mRNA transcript.
5. The type I IGF receptor gene is expressed in lactating mammary tissue. Of the cells in mammary tissue, alveolar epithelial cells contain the highest relative abundance of type I receptor mRNA. GH treatment results in a decrease in the abundance of type I receptor mRNA transcripts.
6. The EGF receptor gene is expressed in lactating mammary tissue, predominantly in alveolar epithelial cells. There is a decrease in the abundance of EGF receptor mRNA as a result of GH injection.

Further experimentation will be required to determine the exact biological relevance of these findings; however, current knowledge about the regulatory molecules provides a basis for interpretation of the findings to substantiate the hypothesis of this thesis. During GH treatment, alveolar epithelial cells are exposed to a set of regulatory molecules that include GH, IGF-I, and EGF. GH probably plays a multifunctional role by 1) altering the rate of IGF-I gene expression in both mammary tissue and nonmammary tissues and 2) by resetting the context of epithelial cell physiology. The action of GH on EGF biology may be realized, at least in part, through changes in EGF receptor biology.

The primary actions of IGF-I, EGF, and probably other unknown regulatory molecules result in proliferation of epithelial cells, stimulation of their functional ability, or both. These actions also produce alterations in the biology of homologous receptors, and possibly heterologous receptors, that are congruent with the physiological context

required for the primary response. Alterations in heterologous receptor biology may, in fact, be essential to setting the context for the primary response.

Changes in mammary cell physiology in turn lead to alterations in the pattern or set of regulatory signals that originate in mammary tissue. These alterations are integrated by an overall regulatory system, which induces appropriate changes in the metabolism of nonmammary tissues. In general terms, the overall regulatory system directs metabolic changes not only during the initial stages of GH treatment, but also continuously as feed intake progressively increases to meet the demands for additional energy and precursors for milk synthesis.

The experimental findings in this thesis provide some of the first insight into the molecular and cellular bases of GH's role in bovine mammary gland biology. Moreover, the realization that GH plays an important role in mammary physiology is now reinforced by the finding that GH induces changes in the biology of several regulatory molecules in mammary tissue. The marked increase in milk production observed during GH treatment, however, is still the most convincing change in this regard.

The practical implication of new insight into GH's role in mammary physiology is that it provides a basis for the development of strategies for future research in this area. The underlying molecular mechanisms responsible for the changes in the biology of the regulatory molecules identified in this thesis certainly warrant further study. Learning the molecular basis and biological relevance of these changes is an important step toward a more complete understanding of GH's role in mammary biology. Another challenge is to identify the other signals associated with GH action on mammary cells.

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APPENDIX 1: SUBCLONING IN LOW MELTING POINT AGAROSE

General Procedure

Day 1 - start overnight bacterial culture (ie. innoculate a single colony into 10 ml Luria-Bertani (LB) medium and grow overnight at 37°C with shaking)

Day 2 - prepare competent cells
- digest vector DNA and DNA to be inserted
- run LMP gel, excise desired fragments
- alkaline phosphatase (if necessary)
- start ligation reaction
- make LB and agar plates with antibiotic

Day 3 - do transformations

Preparation of Competent JM83 and HB101

Mix #1

100 mM NaCl
5 mM $MgCl_2$
5 mM Tris-HCl, pH 7.6

To make 150 ml:

3.0 ml 5 M NaCl
0.75 ml 1 M $MgCl_2$
0.75 ml 1 M Tris-HCl, pH 7.6

- dissolve and then bring to 150 ml with Milli Q (MQ) H_2O.
- autoclave 20 min

Mix #2

100 mM $CaCl_2$
250 mM KCl
5 mM $MgCl_2$
5 mM Tris-HCl, pH 7.6

To make 150 ml

2.2053 g $CaCl_2$
2.7960 g KCl
0.75 ml 1 M $MgCl_2$
0.75 ml 1 M Tris-HCl, pH 7.6

- dissolve and then bring to 150 ml with MQ H_2O.
- autoclave 20 min

Protocol

1. Add 500 µl JM83 (or HB101) *E. coli* overnight culture to 100 ml sterile LB.

2. Incubate at 37°C with shaking (200 rpm) for 2-4 h (ie. until in log phase of growth; I routinely incubate 2.5 h).

3. Aliquote LB containing JM83 (or HB101) into 2 sterile 50 ml Oakridge tubes (ie. 50 ml in each).

4. Centrifuge at 4000 rpm for 5 min (4°C) with NO BRAKE.

5. Decant supernatant.

6. Resuspend pellet in one tube in approx. 45 ml Mix #1 (ie. 1/2 the total original LB volume).

7. Pour the resuspension mixture into the other tube and resuspend the second pellet.

8. Let stand on ice 5 min.

9. Centrifuge at 4000 rpm, 5 min, 4°C, no brake.

10. Decant supernatant.

11. Add 20 ml Mix #2 (ie. 1/5 the total original LB volume) and resuspend pellet.

12. Let stand on ice 40 min.

13. Centrifuge at 4000 rpm, 5 min, 4°C, no brake.

14. Decant supernatant, resuspend pellet in 2 ml Mix #2.

15. Aliquot into sterile eppendorf tubes, 200 µl in each.

16. Allow to stand overnight at 4°C for OPTIMUM TRANSFORMATION EFFICIENCY.

Digests (EXAMPLE) and LMP Gel

1. Vector Digest

11 µl H_20
5 µl Vector DNA (eg. 500 ng, 3000 bp vector)
2 µl 10× reaction buffer
1 µl restriction enzyme #1
1 µl restriction enzyme #2
20 µl

2. Insert Digest

11 µl H_20
5 µl Vector containing insert DNA (eg. 500 ng, 3000 bp vector containing a 700 bp insert)
2 µl 10× reaction buffer
1 µl restriction enzyme #1
1 µl restriction enzyme #2
20 µl

3. Vector and insert digests into 37°C for 1 h.

4. 1% LMP gel: 0.4 g LMP agarose (BRL; Cat. No. 5517 UA)
40 ml 1× TAE
1.5 µl Ethidium Bromide (10 mg/ml stock)

Dissolve agarose and pour into mini-gel with 12 well comb (capacity 20-25 µl/well)

5. When digests complete, add 2 µl gel tracking dye to each.

6. Load DNA, run at 100 V for 10 min, then at 70 V until fragments of interest are resolved enough to excise.

7. Cut appropriate insert and vector fragments out of gel using a new sterile scalpel blade for each. Cut/trim to get small a volume as possible (30-50 µl).

   eg. Vector = 500 ng in 40 µl gel (3000 bp)
   Insert = 95 ng in 39 µl gel (700 bp)

8. Melt gel slices at 70°C for 5-15 min in water bath.

9. Combine an appropriate amount of the vector and insert solutions (approximately 2:1 to 4:1, insert:vector) to give a final volume of 10 µl.

   eg. 1 µl vector gel (12 ng DNA)
   9 µl insert gel (22 ng DNA)
   10 µl

10. Allow mixture to equilibrate to 37°C for 15 min.

11. Add 10 µl ice-cold 2× ligase buffer, containing T4 ligase, mix quickly.

    10× ligase buffer: 400 mM Tris-HCl, pH 7.5
    100 mM $MgCl_2$
    100 mM DTT
    10 mM ATP
    500 µg/ml BSA

    2× ligase buffer/T4 ligase: 15 µl H_20
    4 µl 10× ligase buffer
    1 µl T4 ligase (BRL 1 unit/µl)
    20 µl

12. Incubate (20 µl ligase reaction) at 15°C for 3-24 h (I usually incubate overnight, 18 h).

    Note: Remelt at 70°C before performing transformation (See "Transformation Protocol").

LB/Agar Solution (with Ampicillin) for Plates

To make 500 ml LB + agar: (20-25 plates)

5.0 g Peptone 140
2.5 g yeast extract
5.0 g NaCl

- dissolve above reagents in 450 ml H_20 in a 500 ml beaker
- adjust pH to 7.5 with 10 N NaOH, then 1 N NaOH
- transfer to a 500 ml graduated cylinder and bring to 500 ml with H_20
- add to 6 g agar in a 1 liter Erlenmeyer flask
- autoclave 20 min

- allow to cool for 10-15 min
- add 2.0 ml Ampicillin (Amp) stock (25 mg/ml) to the 500 ml LB/agar solution (final concentration = 100 µg/ml)
- pour plates
- leave at room temperature overnight

Transformation of Ligated Subclones into Competent HB101 or JM83 *E. coli*

1. Prepare LB top

   To make 50 ml:

   - add 50 ml LB to a 250 ml Erlenmeyer flask containing 0.3 g agar
   - autoclave 20 min
   - after autoclaved, keep at 60-70°C (to prevent gelling) until later (see below).

2. Prepare TCM solution (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$)

   For 100 ml:

   1 ml 1.0 M Tris-HCl, pH 7.5
   1 ml 1.0 M $MgCl_2$
   0.147 g $CaCl_2$

   - dissolve and bring to 100 ml in MQ H_2O
   - autoclave 20 min

3. Remelt 20 µl ligation reaction (in LMP) at 70°C for 15 min

4. Prepare the following reactions:

   Reaction #1
   200 µl competent *E. coli* cells
   5 µl ligation reaction

   Reaction #2
   First dilute remaining 15 µl ligation reaction by adding 135 µl ice-cold TCM solution.

   200 µl competent *E. coli* cells
   150 µl diluted ligation reaction

5. Gently mix the above reactions, and place tubes on ice for 30 min.

6. Preheat water bath to 42°C.

7. Place Amp plates in 37°C incubator to warm.

8. Place transformation reaction tubes (in a rack) in 42°C water bath for EXACTLY 2 min.

9. Add 1 ml LB to each tube, gently mix.

10. Incubate at 37°C for 1 h -- NO SHAKING.
    (Amp plasmids for 1 h; Tet plasmids for 30 min)

Note: Approximately 20 min before the 1 h, 37°C incubation is complete, perform the following steps:

(1) Add 3 ml hot (60-70°C) LB top to each of 2 sterile 5 ml tubes (polystyrene or polypropylene; Falcon tubes).
(2) Allow LB top to equilibrate to 42°C for 15 min.
(3) Add 8.4 µl Amp stock (25 mg/ml) to each 5 ml tube containing LB top (final Amp concentration = 50 µg/ml); replace into 42°C water bath.

11. After 1 h, 37°C incubation is completed, take 5 ml tubes (one at a time) out of 42°C water bath, dry off with tissue, and add one transformation reaction to each.

12. Mix tubes by inversion, 2-3 times.

13. Quickly pour solution onto warm Amp plate, and then tilt plate gently around to spread solution evenly over plate.

14. Allow plates to stand at room temperature on bench for 15 min to allow LB top to gel.

15. Place plates in 37°C incubator (agar hanging down). Colonies (transformants) should be visible in 12-16 h.

Characterization of Subclones

Plasmid mini-preps are adequate for verification and characterization of new subclones, or any plasmid DNA. I routinely use the mini-prep protocol described by Miller *et al.* (Methods Enzymol. 152:165, 1987), but start with a 10 ml overnight culture. The DNA obtained using this procedure is adequate for restriction enzyme analysis.

Large-scale Preparation of Plasmid DNA (eg. subclones)

Although mini-preps yield DNA that can be used for further manipulation (eg. probe labeling), it is more convenient to prepare a large amount of "clean" plasmid DNA. Moreover, large-scale preparations are most reliable in terms of success in further manipulation. Large-scale preps are obtained using the CsCl ultracentrifugation procedure described by Maniatis *et al.* (Molecular Cloning, Cold Spring Harbor Laboratory, 1982), except that two CsCl spins are performed instead of only one.

APPENDIX 2: GUANIDINE THIOCYANATE/CsCl PROCEDURE FOR TOTAL RNA ISOLATION

Background

Guanidinium salts are very efficient protein denaturants and thus facilitate the isolation of intact, functional RNA. Their ability to inhibit ribonuclease activity is much greater than that provided by phenol and urea based procedures. In the guanidine thiocyanate (GIT)/CsCl procedure, tissue is homogenized in a GIT buffer and then layered on a CsCl cushion. Physical separation of RNA from other components in the homogenate is achieved by selective sedimentation, using ultracentrifugation, and is based on bouyant density in the CsCl cushion:

(1) Proteins remain in aqueous quanidine region.
(2) DNA bands in the CsCl (and can be recovered).
(3) RNA pellets in the bottom of the tube as a clear, gelatinous or jelly-like pellet (RNA has high bouyant density relative to other components).

The GIT/CsCl procedure gives much higher RNA yields than the lysis buffer/phenol extraction type procedures. Moreover, it is <u>much</u> easier and faster. I have also recently tried an acid GIT/phenol method (Chromczynski *et al.* Anal. Bioch. 162:156, 1987). In my hands, it does not even come close to the ease and efficiency of the GIT/CsCl procedure.

The following GIT/CsCl procedure is based on the original procedure developed by Chirgwin *et al.* (Biochem. 18:5294, 1979).

Tissue Collection Consideration

If you are now at the initial stages of designing an experiment to examine the expression of specific genes in a tissue, in response to some type of physiological manipulation, you should perhaps consider at least one other potentially important question. Would information regarding the site of expression (ie. cell type in a heterogenous tissue) of the gene of interest be valuable in understanding the physiological question your experiment is designed to address? If your answer to this question is, yes, then consider incorporating the technique of *in situ* hybridization into your experimental design. Very little tissue is required (ie. 10-100 mg) for *in situ* hybridization and the tissue preservation steps are fast and simple. After embedding, the tissue can also be stored for several months before performing in situ. Also, many of the techniques required for *in situs* are the same as those for Northerns. Most importantly however, if you have preserved some tissue, then you will at least have the option to perform *in situs*. Without the preserved tissue, the *in situ* option is only available if you repeat your entire experiment!

Avoiding Ribonuclease Contamination

<u>Note</u>: It is <u>critical</u> to the preparation of intact RNA, as well as to subsequent RNA manipulations, that ribonuclease contamination is absolutely avoided. The suggestions below work well for me. I just use common sense, with <u>no</u> compromising.

1. <u>Always</u>, wear gloves when handling anything that has to do with RNA (ie. solution preparation to RNA manipulation, etc.). Fingers are loaded with RNases!

2. Glassware Cleaning:

(1) Ultrasonic water bath with detergent (can also use acid baths, but they are more work).

(2) Distilled H_2O rinse 10 times.

(3) Milli Q (MQ) H_2O rinse 10 times, dry in drying oven, cover with foil.

3. Glassware Baking (also spatulas, dissecting instruments, pipets, etc.):

(1) Everything is clean and covered or wrapped in foil.

(2) Bake at 250°C overnight (12-18 h).
(autoclaving does not destroy all ribonucleases)

4. Sterile MQ H_2O:

(1) Rinse 2 litre acid stock bottles (empty) with MQ H_2O (10-20 times).

(2) Bake bottles without caps; caps are autoclaved.

(3) Fill with MQ H_2O (2/3 full).

(4) Cap loosely and autoclave 40 min on liquid cycle.

(5) Sterile MQ H_2O is used for preparation of <u>all</u> solutions, rinsing, etc.
(I do not treat H_2O or RNA solutions with diethylpyrocarbonate.)

5. Sterile plastic ware (commercially sterilized) is considered RNase free.

6. Use only new reagents, or those specifically designated for RNA use.

- never risk using "ordinary" lab reagents.
- concentrated HCl, NaOH, etc., are taken from lab stocks, but from new bottles; store your own stocks.
- <u>All</u> reagents and solutions are labeled "RNA ONLY".

7. Sterile 50 ml polypropylene tubes with caps are very useful (Cat. No. 25330-50; Corning Laboratory Sciences).

8. Sterile 5 and 10 ml polypropylene or polystyrene Falcon tubes with caps are also useful.

9. 500 ml Gibco bottles are convenient for solution storage. They can be baked and autoclaved (Cat. No. 900-7030). Gibco bottle lids are only autoclaved.

10. Handle reagents with baked spatulas (or "tap" from stock container).

11. Weighing is performed in baked foil weigh dishes.

12. pHing Solutions:

    (1) Calibrate pH meter (ie. use pH 7 and 10, or 7 and 4 solutions).

    (2) Soak electrode in 0.1 N NaOH for 10 min.

    (3) Rinse electrode well with sterile MQ H_20.

    (4) pH solution.

13. All pipet tips and eppendorf tubes are autoclaved only.

14. Educate other lab workers about RNA and RNases!

Equipment

- Autoclaved containers for tissue freezing/storage
- Baked dissection instruments
- Baked foil weigh dishes
- Baked glassware (eg. beakers, volumetric flasks, graduated cylinders, etc.)
- Baked magnetic stir bars
- 0.2 and 0.45 µm Nalgene filters (vacuum filters, sterile)
- Baked 5 and 10 ml glass pipets (disposable)
- Baked pasteur pipets
- Baked 30 ml Corex tubes (wrap and bake in packages of six tubes)
- Beckman polyallomer tubes (13.2 ml) for SW 41 Ti (Cat. No. 331372)
- SW 41 Ti rotor (Beckman)
- Polytron (and medium head)
- Autoclaved Kimwipes
- Autoclaved eppendorfs
- Autoclaved 200 and 1000 µl pipet tips
- Baked mortars and pestles

Reagents

- 10 N NaOH
- CsCl (Ultrapure; BRL)
- EDTA ($Na_2EDTA2H_20$)
- GIT
- Sarcosine (Na-lauryl-sarcosine)
- Trizma Base
- β-mercaptoethanol
- SDS
- Sterile MQ H_20
- Liquid N_2

Tissue Collection for RNA Isolation

1. Autoclave appropriate containers for N_2 tissue freezing and -70°C storage.
   - depending upon tissue sample size, use eppendorfs, Mini Vials, 20 ml scintillation vials, etc.
   - label containers with felt marker ("labels" fall off in N_2)

2. Dissect tissues using "sterile technique" as practical.

   (1) When collecting tissue for RNA isolation try to minimize time from sacrificing animal to freezing tissue.

   (2) Use baked instruments when practical. (Do not use baked scissors to cut through hair and skin, and then use the same scissors to cut a piece of liver for RNA isolation. Rather, use "normal" scissors to get into abdomen, and then use baked scissors or sterile scalpel blade to cut liver.)

   (3) Hair is loaded with RNases! (avoid getting hair on tissue samples)

   (4) Handle tissue sample with baked tweezers.

3. Place tissue sample in autoclaved container and drop in N_2. It is easiest to weigh fresh tissue (as opposed to frozen), but weigh in baked foil weigh dish, and try to be fast.

4. Long-term tissue storage is at -70°C.

Solutions for RNA Isolation

1. CsCl (1.7 g/ml)

   5.7 M CsCl
   0.1 M EDTA
   pH 7.0

   To make 100 ml:

   95.97 g CsCl
   3.8 g $Na_2EDTA\,2H_2O$

   - dissolve in less than 100 ml volume sterile MQ H_2O
   - pH to approx. 7.0 with 10 N NaOH, then 0.1 N NaOH
     (original pH approx. 5, need approx. 200 µl 10 N NaOH to reach pH 7)
   - transfer quantitatively to a baked 100 ml volumetric flask
   - bring to 100 ml with sterile MQ H_2O, mix
   - filter sterilize with 0.2 µm Nalgene filter
   - store at room temperature (in sterile 50 ml tubes)

2. GIT

   4 M guanidine isothiocyanate
   0.5% Na-lauryl-sarcosine
   25 mM Tris-HCl, pH 7.5
   0.1 M β-mercaptoethanol

   To make 100 ml:

   CAUTION: GIT is very toxic. Weigh and make GIT solution in fume hood. Wear lab coat, eye protection, and rubber gloves.

   47.28 g GIT
   0.5 g Na-lauryl-sacosine
   2.5 ml 1 M Tris-HCl, pH 7.5
   700 µl β-mercaptoethanol (stock = 14.3 M)

- dissolve GIT and sarcosine with stirring in MQ H_2O at 60°C (add H_2O to GIT in beaker to just less than 100 ml)
- no need to pH this solution
- bring to 100 ml with H_2O
- filter with 0.2 µm Nalgene filter
- store at room temperature (in sterile 50 ml tubes), only 1-2 weeks

3. 1 M Tris-HCl, pH 7.5

To make 500 ml:

60.55 g Trizma base

- dissolve in 400 ml MQ H_2O
- add 25 ml concentrated HCl
- allow to cool to room temperature
- make final pH adjustment to pH 7.5 with concentrated HCl (may have to add another 2-5 ml concentrated HCl)
- bring to 500 ml with MQ H_2O
- autoclave 20 min (in a 500 ml Gibco bottle)

4. 0.1 N NaOH (for "RNasing" polyallomer tubes and pH electrode)

To make 50 ml:

500 µl 10 N NaOH

- bring to 50 ml with MQ H_2O (in a 50 ml polypropylene tube)

5. SET Buffer (for dissolving RNA pellets)

10 mM Tris-HCl (pH 7.5)
5 mM EDTA
0.1% SDS

To make 50 ml:

500 µl 1 M Tris-HCl (pH 7.5)
500 µl 0.5 M EDTA (pH 8)
500 µl 10% SDS

- bring to 50 ml with MQ H_2O (in a 50 ml polypropylene tube)

6. 0.5 M EDTA, pH 8.0

To make 500 ml:

93.05 g $Na_2EDTA2H_2O$

- add to 400 ml H_2O, stir vigorously with a baked magnetic stir bar
- add 10 N NaOH while monitoring pH (EDTA will not dissolve until pH is almost 8)
- add MQ H_2O to just less than 500 ml, continue mixing
- if still not dissolved, heat
- make final pH adjustment to 8.0 with 10 N NaOH
- bring to 500 ml with H_2O
- autoclave 20 min (in 500 ml Gibco bottle)

- store at room temperature

7. 10% SDS

To make 500 ml:

50 g SDS

- add to 450 ml H_2O, mix with baked magnetic stir bar
- heat to 65°C to aid dissolution
- bring to 500 ml with H_2O
- no need to pH or sterilize

RNA ISOLATION PROCEDURE (SW 41 Ti Rotor)

Set-up

1. Cool baked mortars and pestles at -70°C.

2. Treat 6 polyallomer tubes with 0.1 N NaOH (5 min) and rinse well with sterile MQ H_2O.

3. Transfer 3.3 ml CsCl solution to each tube (using a baked 5 ml pipet) and cover tubes with foil (3.3 ml is 1/4 full).

4. Transfer 10 ml GIT solution to each of 6 baked 30 ml Corex tubes.

Note: This volume may be varied to maximize RNA obtained from tissue. Some homogenate solution is usually lost after spinning out cell debris (see below) because it must be sacrificed to avoid contaminating the homogenate solution with cell debris.

Tissue Pulverization

5. Pulverize tissue (pre-weighed to 0.5-1 g) in a pre-chilled mortar (-70°C) containing liquid N_2. Pulverize to fine powder.

Notes:
(1) Each of the six tubes can handle up to 1 g tissue.
(2) Normally, use 6 separate baked mortars and pestles, one for each sample. However, if pooling samples or using 6 g tissue from one animal, then pulverize using one mortar and pestle.
(3) Transfer frozen powder to a frozen tube (15 ml polypropylene). This facilitates transfer (see below) to 30 ml Corex tubes containing GIT solution.
(4) Before starting tissue homogenization, pulverize all 6 samples (and transfer to frozen tubes).

Tissue Homogenization

6. Quickly add frozen tissue powder to GIT solution.

7. Immediately homogenize tissue at full speed for 60 sec at room temperature.

Notes:
(1) Use medium sized polytron probe (1 cm diameter).
(2) If excess foaming occurs by 30 seconds, centrifuge Corex tube at 10,000 rpm for 2-3 min, then homogenize for remaining 30 sec.

8. Homogenize all samples, rinsing polytron probe in a large volume of MQ H_20 (ie. 1 litre, 2 times) between samples.

9. To pellet cell debris, centrifuge homogenate in Corex tubes at 6500 rpm (5000 g) for 10 min at 4°C.

   Note: Some protocols state that the homogenate can be frozen, and ultracentrifuged later.

Layering Homogenate on CsCl

10. After centrifugation, carefully remove Corex tubes from centrifuge (so not to disturb cell debris pellet).

11. Carefully remove homogenate from each tube with a baked 10 ml pipet (try not to disturb or remove any cell debris pellet), and very carefully layer as much as possible on the 3.3 ml CsCl cushion in each polyallomer tube.

    Note: If possible, fill polyallomer tubes to 2 mm from top of tube. If recovery volume is not sufficient, then top up tubes with GIT solution. It is critical to fill tubes to prevent collapse during ultracentrifugation.

Ultracentrifugation: tubes, buckets, and rotor

12. Carefully place tubes in rotor buckets and install caps, tightening caps only until they just ground on metal. Overtightening caps makes removal difficult and increases risk of damage to caps. If the caps are too loose, the vacuum in the ultracentrifuge will remove your homogenate!

13. Mount buckets on rotor - PROPERLY.

    Notes: (1) See rotor manual for correct bucket-rotor position and for rotor balancing if using less than six full tubes.
    (2) Mount buckets on rotor at ultracentrifuge to avoid sample mixing on the way there.

Ultracentrifuge: Operation (SW 41 Ti Rotor; 30,000 rpm, 23 h, 20°C)

14. Turn on power.

    (1) Diffusion pump AUTO
    (2) Refrigeration OFF
    (3) Temperature limit at 30°C
    (4) Temperature set at 20°C

    Note: After starting some ultracentrifuges the temperature drops considerably. If you are using such an ultracentrifuge then initially set the temperature at 25°C. After the temperature has stabilized, (approx. 25 min), reset it to 20°C.

15. Load rotor with buckets/tubes (refrigeration still OFF). Close door.

16. Turn on vacuum (refrigeration still OFF).

17. When vacuum reaches 200 μ, turn on ultracentrifuge by pressing start button (1-3 sec) until RUN light remains on (timer on HOLD, speed at 30,000 rpm). Also, turn on refrigeration. Temperature set at 20°C (or 25°C).

18. Start timing the run 10 min after starting rotor (ie. it takes about 10 min to reach 30,000 rpm). If necessary make final speed adjustment to achieve 30,000 rpm.

19. Turn off ultracentrifuge 23 h after it has reached 30,000 rpm.

Notes on Ultracentrifugation:

(1) Do not allow the ultracentrifuge temperature to fall below 20°C or CsCl may precipitate and effect the final quality and yield of RNA.
(2) If CsCl precipitates, then it must be removed by further purification steps (see below). Further purification is also required if the capacity of the CsCl cushion is exceeded (eg. if more than 1 g tissue was in homogenate).
Note: Normal, clean RNA pellets are clear and gelatinuous. Contaminated RNA pellets are granular or discolored.
(3) Further purification of contaminated pellets:
(i) Dissolve pellet in 0.3 M Na-acetate, pH 6.0.
(ii) Extract with equal volume phenol/chloroform (buffered).
(iii) Centrifuge 5 min, remove upper aqueous phase.
(iv) Add 0.1 volume 3.0 M Na acetate and 2.5 volume 95% ethanol (-20°C).
(v) Precipitate overnight at -20°C or at -70°C for at least 30 min.
(vi) Microfuge at 4°C, 30 min (or at 10,000 rpm in Corex).
(vii) Remove supernatant and wash pellet with cold 80% ethanol (-20°C), (ie. vortex briefly with 1-2 ml ethanol).
(viii) Microfuge at 4°C, 10 min (or at 10,000 rpm in Corex).
(ix) Remove supernatant, air dry pellet.

Recovery of RNA Pellet after CsCl Ultracentrifugation

20. After ultracentrifuge has come to a complete stop, turn off vacuum. When vacuum drops to zero, open door and carefully remove rotor with buckets. Also, carefully remove bucket caps.

21. Carefully, remove most of the supernatant by aspiration with a baked pasteur pipet, leaving 1-2 ml CsCl in tube.

22. Carefully remove remaining solution by aspiration with a fresh baked pasteur pipet. Do not disturb the gelatinuous RNA pellet. Turn tubes upside down and set on tissue paper to drain.

23. Remove residual droplets using sterile Kimwipes (without introducing finger RNases).

Dissolving RNA Pellet

Important Note: This is perhaps the most difficult and critical step of the whole isolation procedure. Low yields of RNA are usually the result of difficulties encountered dissolving recalcitrant pellets. Several published protocols provide strategies to overcome this problem, with some being more harsh than others. I generally start with mild treatments (ie. vortexing and pipeting) and then proceed to harsher treatments (ie. heating) if difficulty with dissolution persists.

24. Determine an appropriate resuspension volume.

Considerations:

(1) Desired RNA solution concentration.
(2) Use of RNA preparation only for total RNA gels.
(3) Use of RNA preparation for isolation of poly(A)$^+$ RNA.
(4) Dissolve RNA at approx. 6 mg/ml. At this concentration, up to 30 µg of total RNA can be easily loaded in a lane/well on a gel (without having to lyophilize, ethanol precipitate, or dry down your RNA samples).

Examples of approximate yields:

(1) Bovine mammary tissue (lactating) 2.5 mg RNA/g tissue
(2) Rat/rabbit/bovine liver 3.5-4.0 mg/g
(3) Human placenta 1 mg/g
(4) Rat brain 200 µg/g
(5) Rat/bovine skeletal muscle 300 µg/g

25. Resuspend RNA pellet in 3/4 of final resuspension volume of SET buffer (will use other 1/4 volume to rinse tube). Resuspend by pipeting and vortexing.

Note: Even extensive pipeting and vortexing at this stage may not result in a completely dissolved pellet; nevertheless, proceed to step 26.

26. Transfer RNA solution (still in only 3/4 volume SET, and possibly with RNA "pieces") to a sterile eppendorf tube.

27. Add 1/4 volume (of final resuspension volume) of SET to the original polyallomer tube, vortex. Transfer this rinse to the RNA solution in the eppendorf tube.

Dissolving RNA Pellet, COMPLETELY

28. If small, clear chunks of RNA persist:

(1) subject RNA solution to one freeze (-70°C) -thaw cycle, vortex.
or, (2) heat RNA solution at 65°C for 5 min, vortex.
or, (3) perform (1), followed by (2).

Note: The treatment(s) that you subject your RNA solution to, should be based on how dissolved it is before you freeze or heat it (ie. often pipeting and vortexing will dissolve the RNA pellet, so do not unnecessarily freeze or heat it). Also, depending on what you will be doing with your RNA, it may be necessary to freeze or heat it anyway (eg. poly(A)$^+$ RNA isolation).

Estimation of RNA Concentration and Purity

29. RNA concentration is estimated by absorbance at 260 nm on the spectrophotometer. RNA purity is estimated by the 260/280 nm ratio. (260/280 = 2.0 = clean RNA)

Notes: (1) Add 2-4 µg RNA to 1 ml H_2O (ie. cuvette volume for spectrophotometer) to obtain a reliable reading.
(2) Ensure that your RNA is completely dissolved before attempting spectrophotometry.

30. Your dissolved RNA is sufficiently pure for dot blots, Northerns, and poly(A)$^+$ RNA isolation.

RNA Storage and Aliquoting

31. Store RNA in SET buffer at -70°C.

Note: Whenever possible, it is best to aliquot your RNA preparation in appropriate volumes/amounts. For example; if you plan to run only total RNA gels, and require 30 µg RNA to detect the transcript of interest, then aliquot your entire preparation into 30 µg aliquots (regardless of volume). Aliquoting avoids unnecessary and damaging freeze-thaw cycles.

APPENDIX 3: ISOLATION OF POLYADENYLATED RNA

Background

The majority of cellular RNA is ribosomal and transfer RNA. Only 1 to 3% of RNA is polyadenylated [poly(A)$^+$] messenger RNA. Poly(A)$^+$ RNA is isolated from total cellular RNA to (1) generate cDNA clones by reverse transcription and second strand synthesis, (2) perform *in vitro* translation, or (3) to facilitate detection of low abundance mRNAs. By virtue of the 3′ poly(A)$^+$ tract on mRNA, it is readily separated from total RNA using oligo d(T) cellulose chromatography.

The chromatography procedure is based on the ability to stabilize or destabilize A-T hybrids by changing the salt concentration of chromatographic buffers. One of two different salts (NaCl or LiCl) are used for the buffer system.

My initial efforts in isolating poly(A)$^+$ RNA were devoted to the optimization of the procedure using NaCl-based buffers. Although this buffer system can be successfully employed, it is more difficult to use, and therefore less reliable than the LiCl-based buffer system. The major difficulty encountered with NaCl buffers is impeded or variable column flow. This is due to the low solubility of SDS in the high concentration Na buffers. Omission of SDS solves this problem, but generally results in a higher level of ribosomal RNA contamination in the final poly(A)$^+$ RNA preparation.

Since LiCl inhibits the reverse transcriptase enzyme, which is used to generate cDNA molecules from poly(A)$^+$ RNA, the NaCl buffer system should be used in this application. In this instance, keep the NaCl binding buffer at 37°C throughout the procedure to minimize column flow problems. It is also critical to thoroughly wash the column (ie. use large volumes of binding buffer) at each wash or binding step. The input total RNA should also be used at low concentration (1 mg/ml or less).

Even with these modifications to the NaCl system, it is not recommended for poly(A)$^+$ RNA isolation when the purpose is to make quantitative comparisons between RNA samples. In fact, even using the LiCl system it is critical to "standardize" the column procedure in order to make meaningful quantitative comparisons. In general terms, "standardization" is accomplished by (1) using exactly the same size column for each RNA sample, (2) preparing each column in the same manner, (3) using the same amount and concentration of input RNA for each sample, (4) treating each RNA sample in exactly the same manner, and (5) using the same buffer volumes for each sample at each step. The importance of these considerations can not be overemphasized.

Solutions

1. 1 M Tris-HCl, pH 7.5 (autoclaved)
2. 0.5 M EDTA, pH 8.0 (autoclaved)
3. 10% SDS (prepare using sterile H_20, do not autoclave)
4. 3 M Na acetate, pH 5.5 (autoclaved)
5. 95% ethanol
6. 0.1 N NaOH/5 mM EDTA (autoclaved)
7. TE, pH 7.6 (10 mM Tris-HCl, 1 mM EDTA) (autoclaved)
8. 10 M LiCl (filter using 0.45 µm Nalgene vacuum filter)
9. Elution buffer: 10 mM Tris-HCl, pH 7.5
2 mM EDTA
0.1% SDS (add SDS after autoclaving)
10. Binding buffer: 0.5 M LiCl
10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.1% SDS (add SDS after autoclaving)

11. Wash buffer: 0.15 M LiCl
10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.1% SDS (add SDS after autoclaving)

Equipment

- Oligo d(T) type 3 (Collaborative Research)
- Disposable polypropylene columns, autoclaved (Biorad; Cat. No. 731-1550)
- sterile 5 and 15 ml Falcon tubes with caps (polypropylene or polystyrene)

General Considerations

1. When it is necessary to process many samples, it is efficient to run up to 4 columns at the same time. However, before running several columns simultaneously it is best to have already established <u>your own</u> "standard" of running the protocol.

2. One round of oligo d(T) means passing input total RNA through the column 3 times (washing with binding buffer after each pass), washing with "wash buffer", and eluting poly(A)$^+$ RNA. For two rounds, this process is repeated using the poly(A)$^+$ eluted after the first round.

3. Two rounds of oligo d(T) result in the highest enrichment of poly(A)$^+$ in the final RNA preparation. However, with small amounts of input total RNA only one round is possible. This limitation is mostly due to the requirement for RNA to estimate the concentration of the RNA recovered. At least 1-3 μg of RNA is required to obtain a reliable spectrophotometer reading. Thus, the lower limit of input total RNA is around 300 μg.

4. Two rounds of oligo d(T) are usually performed when input total RNA is not limiting. The best results are obtained when two separate columns are used, one for each round. The first column is prepared using 0.15 g dry oligo d(T) for every 5.0 mg of input total RNA. The second column is prepared using 0.05 g dry oligo d(T) for every 5.0 mg of input (ie. starting) total RNA. It is important to scale the amount of oligo d(T) according to the amount of input total RNA. [0.25 g dry oligo d(T) yields approximately 1.0 ml buffer-swollen oligo d(T)].

5. Unlike many other chromatography procedures, it is not necessary to prevent the oligo d(T) column from "drying out". In fact, with each application of buffer to the column, the column is allowed to drain (drip) until it stops dripping.

Column Preparation

1. Be gentle with oligo d(T) cellulose.

2. Weigh appropriate amount of dry oligo d(T) in a baked foil weigh dish.

3. Transfer dry oligo d(T) to a 5 or 15 ml sterile tube.

   <u>Note</u>: Since total RNA preparations are at -70°C, it is convenient to remove them at this step, and place them on ice to thaw.

4. Add 2 ml elution buffer (EB) for every 0.1 g dry oligo d(T) and slowly (gently) invert tube several times to suspend oligo d(T).

5. Allow oligo d(T) to settle briefly (1-2 min) and then aspirate the top layer of EB containing "fine" particles of oligo d(T).

6. Repeat steps 4 and 5, three more times.

7. The oligo d(T) is poured, as a slurry in EB into the column.

8. Wash the oligo d(T), in the column, with 10 column volumes of binding buffer (BB).

   Note: If the column is prepared with "used" oligo d(T), then the first wash is with 0.1 N NaOH/5 mM EDTA. Following this treatment, wash with sterile Milli Q (MQ) H_20 until the effluent is less than pH 8.0 (use pH paper). Finally, wash with 10 column volumes BB.

9. The column is now ready for application of RNA.

Column Procedure

1. Determine the exact volume of the input total RNA solution (1-2 mg RNA/ml in SET, TE, or H_20).

2. Heat RNA solution at 65°C for 10 min in a water bath. Heating denatures the RNA and thus promotes the formation of A-T hybrids.

3. Add 10 M LiCl to make RNA solution 0.5 M LiCl (ie. add 1/20 volume 10 M LiCl), mix.

4. Gently apply RNA solution to column. Collect all eluate into a 5 or 10 ml sterile tube.

5. When RNA solution is completely through the column (ie. column stops dripping), wash column with 2 column volumes BB. Rinse sides of column when adding this BB wash. Collect the eluate (BB) into the eluate tube of step 4.

6. Load all eluate onto the column, collect, and again wash with 2 column volumes BB.

7. Repeat step 6 one more time.

   Note: The eluate from this step should be saved and the RNA [poly(A)$^-$] precipitated. Poly(A)$^-$ RNA serves as a control (on Northerns) to evaluate the level of nonspecific probe interaction with ribosomal RNAs.

8. Elute poly(A)$^+$ RNA with 2 column volumes EB. Collect eluate into a 5 ml tube (or collect fractions as outline in "note" below).

   Note: With small amounts of input total RNA (eg. 300-600 µg) it is necessary to collect this eluate in fractions, 2-3 drops each, into eppendorf tubes. Fractions containing poly(A)$^+$ RNA are identified by pipeting 2 µl of each fraction into wells made in an ethidium bromide/agarose plate:
   - 0.4 g agarose
   - 40 ml TE

- dissolve agarose by heating
- add 4 µl ethidium bromide (10 mg/ml)
- pour into a petri dish, allow to gel
- aspirate wells using a pasteur pipet
- about 15 min after loading wells, view plate by UV light.
- pool RNA containing fractions, add 1/10 volume 3 M Na Acetate (pH 5.5) and 2.5 volumes cold (-20°C) 95% ethanol
- incubate overnight at -20°C
- microfuge for 30 min at 4°C
- aspirate supernatant, air dry pellet (5-15 min), and resuspend RNA in TE

9. Before performing a second round of poly(A)$^+$ RNA selection, determine <u>exact</u> volume of eluate obtained in step 8.

10. Heat RNA solution at 65°C for 10 min in a water bath.

11. Add 10 M LiCl to make RNA solution 0.5 M LiCl, mix.

12. Repeat steps 4 to 8.

    <u>Note</u>: Remember to scale-down all buffer volumes appropriately in the second round; the second round column contains less oligo d(T), and much less RNA is being processed.

13. To the final eluate [containing poly(A)$^+$ RNA] add 1/10 volume 3 M Na acetate and 2.5 volumes cold (-20°C) 95% ethanol.

14. Incubate overnight at -20°C.

15. Microfuge for 30 min at 4°C.

    <u>Notes</u>: (1) Large volumes of eluate can be aliquoted into several eppendorf tubes. Alternatively, process RNA in either baked, silanized Corex tubes or sterile 15 ml polypropylene tubes. Both types of large tubes are centrifuged at 10,000 rpm for 30 min (4°C).

    (2) RNA can be quantitatively recovered by ethanol precipitation from solutions containing as little as 1 µg/ml RNA. With lower concentration RNA solutions, ultracentrifugation or carrier addition is required for RNA recovery.

16. Aspirate supernatant and air dry pellet for 10-15 min.

17. Resuspend poly(A)$^+$ RNA in TE at 3-6 mg/ml.

18. Aliquot poly(A)$^+$ RNA and store at -70°C. Aliquoting avoids unnecessary freeze-thaw cycles.

APPENDIX 4: RNA GEL ELECTROPHORESIS AND NORTHERN TRANSFER

Standard Gel

Dimensions = 13 x 12 x 0.6 cm (L x W x H).
16 wells with approx. 35 µl capacity each.

Cleaning Electrophoresis Equipment

Designate sets of electrophoresis equipment for RNA use only. Prior to each use, soak electrophoresis unit, trays, combs, etc. in 0.1 N NaOH for 10 min. Rinse thoroughly with sterile Milli Q (MQ) H_2O.

Solutions for Gel Electrophoresis and Northern Transfer

1. 10 x MOPS

   0.2 M MOPS
   50 mM Na Acetate
   10 mM EDTA

   To make 100 ml:

   4.18 g MOPS
   0.68 g Na Acetate $3H_2O$
   0.37 g Na_2 EDTA $2H_2O$

   - dissolve in less than 100 ml sterile MQ H_2O
   - adjust pH to 7.0 with 10 N NaOH
   - bring to 100 ml with sterile MQ H_2O
   - autoclave 20 min
     Note: Solution turns yellow from autoclaving, but this does not effect its performance.
   - store at room temperature covered with foil

2. 10% Bromophenol Blue

   To make 1.0 ml:

   0.1 g bromophenol blue

   - dissolve in 1.0 ml sterile MQ H_2O by vigorous stirring (baked magnetic stir bar) in a baked 20 ml glass scintillation vial.
     Note: Bromophenol blue will not dissolve completely, but the solution will become saturated.
   - store at room temperature

3. 10 mg/ml Ethidium Bromide

   CAUTION: Ethidium bromide is mutagenic. Handle carefully in fume hood, wearing lab coat, gloves, and eye protection.

   To make 10 ml:

   - weigh 100 mg ethidium bromide into a baked 20 ml glass scintillation vial
   - add 10 ml sterile MQ H_2O
   - mix until dissolved (eg. secure vial to a shaker and agitate 1-2 h)

- store at 4°C in a dark bottle or covered with foil

4. 1.0 mg/ml Ethidium Bromide (*CAUTION:* mutagenic)

   - add 1.0 ml 10 mg/ml ethidium bromide to 9.0 ml sterile MQ H_2O, mix
   - store at 4°C in a dark bottle or covered with foil

5. RNA Sample Loading Buffer

   To make 15 ml:

   1.7 ml sterile MQ H_2O
   1.5 ml 10 × MOPS
   7.5 ml 100% deionized formamide
   1.0 ml glycerol (Ultrapure; BRL)
   0.8 ml 10% bromophenol blue
   2.5 ml 37% formaldehyde (12.3 M stock)

   - mix well, and again just before using
   - make fresh weekly or store aliquots at -20°C

6. 10 × SSC

   1.5 M NaCl
   0.15 M trisodium citrate

   To make 1000 ml:

   87.6 g NaCl
   44.1 g Na_3 citrate$2H_2O$

   - dissolve in approx. 900 ml sterile MQ H_2O
   - adjust to pH 7.0 with a few drops concentrated HCl
   - bring to 1000 ml with sterile MQ H_2O
   - 10 × SSC is not autoclaved, but stored in baked glass bottles at room temperature

7. Electrophoresis Buffer (1 × MOPS)

   To make 1000 ml:

   100 ml 10 × MOPS

   - bring to 1000 ml with sterile MQ H_2O

Gel Preparation (1-1.5% agarose, 0.66 M formaldehyde)

In a baked 250 ml Erlenmeyer flask:

1. Add 1-1.5 g agarose.
2. Add 10 ml 10 × MOPS.
3. Add 85 ml sterile MQ H_2O.
4. Dissolve agarose completely (heating and swirling flask over a flame, or in a microwave oven).

5. Allow gel solution to cool to 50-60°C in a water bath.

   Note: If necessary, remove a small amount of hot gel solution to seal gel unit.

6. In fume hood, add 5.4 ml 37% formaldehyde and then gently mix by swirling (avoid creating bubbles).

7. Immediately pour gel solution into gel unit with gel comb in place. Any bubbles are quickly removed with a pipet.

8. Allow gel to stand for 1 h before use.

RNA Gel Electrophoresis

1. Add 25 µl loading buffer to each 5 µl RNA sample (maximum 30 µg RNA in 5 µl).

   Notes: (1) Convenient size markers are the RNA ladder (0.24-9.5 kb) from BRL. Treat marker exactly as test RNA samples.
   (2) Also run poly(A) RNA and, if possible, RNA obtained from a tissue shown to possess the RNA of interest.

2. Mix samples, microfuge briefly, and heat at 65°C for 15 min.

3. Chill RNA samples on ice for 3-5 min.

   Note: Before loading gel, flush wells with electrophoresis buffer. It is not necessary to pre-run agarose gels.

4. Carefully load RNA samples into gel wells.

5. Turn on power and run gel at 100 V (constant voltage) for 10 min, and then at 40 V for 10 h.

6. Carefully remove gel from electrophoresis unit at end of run to view and photograph on UV transilluminator. Place a ruler beside the gel during photography.

Northern Transfer

1. Prepare the gel for transfer by soaking it in two changes (20 min each) of 10 × SSC, with gentle agitation.

   Note: Use baked glass baking trays (Pyrex brand; "Storage Plus") for gel soaking.

2. While gel is soaking, cut nylon transfer membrane (Zeta Probe; Bio-Rad) and 3MM Whatman filter paper to appropriate sizes:

   (1) Cut membrane to the same size as the "final" gel dimensions. Also, cut a small nick in the membrane so that the orientation of the lanes of RNA are discernable after transfer.
   Note: The RNA ladder lane as well as any empty lanes are trimmed off gel using a sterile scalpel blade. It is also convenient to cut the gel along the front of the wells. As a result, after RNA transfer,

one edge of the membrane will represent the origin of electrophoresis.

(2) Soak Zeta Probe membrane for 5 min in H_20, then 5 min in 10 × SSC, just before use.

(3) Cut 9 pieces of 3MM filter paper; 6 are cut to the same size as the "final" gel dimensions, and the other 3 are cut to the same size as the transfer sponge.

3. Just before the last gel soak is complete, soak transfer sponge with 10 × SSC.

Notes: (1) transfer sponges (approx. 3 × 15 × 15 cm) are made from foam mattress pads, obtained from a camping equipment store (eg. Canadian Tire). They are easily cut to size using a band saw.
(2) Transfer sponges are initially cleaned by soaking overnight in detergent water. They are then thoroughly rinsed with MQ H_20, and then with sterile MQ H_20. Sponge rinsing is performed by hand, wearing rubber gloves. After each use, sponges are rinsed thoroughly with MQ H_20. Further, after sponges have been used once, it is only necessary to rinse with sterile MQ H_20 just before using again.
(3) To soak sponge in 10 × SSC, place sponge in a baked glass tray and pour 10 × SSC over sponge, until 10 × SSC in tray is approx. half way up side of sponge.

4. Place the three sponge-size pieces of 3MM filter paper on the soaked (10 × SSC) sponge, pour on a small volume of 10 × SSC, and roll out any bubbles using a baked glass pipet. Pour on more 10 × SSC.

5. Gently remove gel from last 10 × SSC rinse and place gel (upside down) on top (centered) of the sponge/3MM assembly.

Note: It is important to standardize the procedure of transfer set up so that the gel orientation is consistent for all transfers. This is necessary in order to identify RNA samples on final autoradiograms.

6. Pour a small volume of 10 × SSC on top of gel and roll out any bubbles.

7. Pour on more 10 × SSC and place the membrane on the gel. Handle the membrane only with baked tweezers. Roll out any bubbles and flood the membrane with 10 × SSC.

8. Place the 3 gel-size 3MM filter papers on top of gel. Roll out bubbles and pour on more 10 × SSC. Position the remaining 3 filter papers and roll out bubbles.

9. Parafilm is placed on the exposed sponge area, around each side of the gel.

10. Place paper towels (Kimtowels; Kimberly-Clark) on top of assembly to make an absorbant pad about 5-10 cm thick.

11. Place a glass or metal plate on top of absorbant pad and approx. 500 g of weight on the plate.

12. Cover whole assembly with plastic wrap to prevent evaporation.

13. Allow RNA to transfer overnight, or at least 8-10 h.

14. After the RNA has transfered to the membrane, disassemble the transfer apparatus down to the membrane. Remove the membrane using baked tweezers and place it between two pieces of 3MM paper. Allow to air dry 30-60 min, and then bake *in vacu* at 80°C for 2 h.

15. Baked membranes are stored in heat-sealed plastic bags at 4°C.

APPENDIX 5: PROBE LABELING AND NORTHERN HYBRIDIZATION

Probes

This protocol has been optimized for use with riboprobes (antisense RNA probes). Although the protocol also works with cDNA probes, several modifications are required. However, because much higher sensitivity is obtained using riboprobes, it is generally worthwhile subcloning cDNA, contained in "conventional" plasmids, into transcription capable plasmids for riboprobe generation (see APPENDIX 1).

General Procedure

Day 1
- start formamide deionization
- start riboprobe labeling reaction
- filter deionized formamide
- start prehybridization
- purify labeled riboprobe
- start hybridization (end prehybridization)

Day 2
- end hybridization
- wash membrane
- start autoradiography (first exposure)

Day 3
- normally, develop first exposure autoradiogram
- if necessary, start second exposure

Reagents for Riboprobe Preparation

It is critical that all solutions/reagents used for riboprobe preparation are of the highest quality and absolutely free of RNase. For this reason the reagents are purchased commercially, usually in kits, from Promega, BRL, or Bio-Can.

- sterile deionized H_2O
- 5 x transcription buffer [0.2 M Tris-HCl, pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine-$(HCl)_3$]
- 100 mM dithiothreitol
- ribonuclease inhibitor (RNasin; 25-40 units/µl)
- 10 mM each ATP, CTP, GTP, and UTP
- SP6 and T7 RNA polymerase (15-50 units/µl)
- [^{32}P]CTP (1 mCi, 40 mCi/ml, 800 Ci/mmol; Cat. No. NEG-008C, New England Nuclear)
- linerarized plasmid DNA containing cDNA insert of interest
- RQ 1 DNase (Promega)

Reagents/Equipment for Riboprobe Purification

- 1 ml sterile disposable syringes
- 15 ml conical centrifuge tubes (eg. graduated Sarstedt tubes from Fisher)
- baked glass wool
- sterile TE (pH 8.0)
- baked tweezers
- baked pasteur pipets (long)
- phenol/chloroform/isoamylalcohol solution (25:24:1) (phenol is buffer equilibrated with Tris-HCl to pH 8.0)
- chloroform/isoamylalcohol solution (24:1)

Sephadex G-50 Swollen in TE

5 g Sephadex G-50 (Pharmacia; DNA grade, fine)

- add Sephadex to a baked autoclavable bottle
- add 150-200 ml sterile TE (pH 8.0)
- autoclave 15 min (liquid cycle)
- aspirate most TE, replace with 100-150 ml fresh TE
- store at 4°C

Reagents for Prehybridization and Hybridization

1. 20 x SSPE (pH 7.4)

   3.6 M NaCl
   0.2 M sodium phosphate
   20 mM EDTA

   To make 250 ml:

   52.60 g NaCl
   7.80 g $NaH_2PO_4 2H_2O$
   1.86 g $Na_2EDTA 2H_2O$

   - dissolve in approx. 200 ml sterile Milli Q (MQ) H_2O
   - adjust pH to 7.4 using 10 N NaOH (approx. 1.5 ml)
   - bring to 250 ml with MQ H_2O
   - autoclave 20 min
   - store at room temperature

2. 100% Deionized Formamide (prepared fresh for each hybridization)

   To make 100 ml:

   100 ml formamide (Fluka)
   10 g mixed-bed resin (Bio-Rad AG 501-X8)

   - add resin to a baked 250 ml Erlenmeyer flask
   - add 100 ml formamide
   - wrap flask completely with aluminum foil
   - stir gently for 1 h on a shaker
   - filter by vacuum through a sterile 0.45 µm (or 0.2 µm) Nalgene filter covered with foil. Filtration takes 20-40 min
   - store at room temperature covered with foil until needed

3. 50% Dextran Sulphate

   To make 40 ml:

   20 g dextran sulphate (Pharmacia)

   - add dextran sulphate to a sterile 50 ml graduated polypropylene centrifuge tube (with cap)
   - add sterile MQ H_2O so that volume is just less than 40 ml
   - cap, shake tube in shaking incubator at approx. 50°C for 20-30 min
   - when dissolved, bring to 40 ml with MQ H_2O

- 50% dextran sulphate is not filtered or autoclaved
- aliquot, using baked 5 or 10 ml pipets (eg. 2, 3, 4, and 5 ml aliquots are conveniently placed in 50 ml tubes)
- store at -20°C

4. 10% Blotto (low-fat milk powder)

To make 10 ml:

1 g low-fat milk powder (Carnation, Safeway)

- add 1 g milk powder to a sterile 50 ml tube
- add 10 ml sterile MQ H_2O
- mix until dissolved
- make fresh daily, or store at -20°C

5. 20% SDS (APPENDIX 2)

6. Sheared salmon testes DNA (ssDNA; 10 mg/ml)

First, make 10 ml 1 N NaOH (1 ml 10 N NaOH + 9 ml sterile MQ H_2O)

Then, make 20 ml 2 M Tris (4.844 g Trizma base dissolved in 20 ml sterile MQ H_2O; not pH'd)

To make 42 ml ssDNA:

420 mg DNA (Pharmacia; Cat. No. 27-4564-01)

- add DNA to 7.0 ml 1 N NaOH in a sterile 50 ml polypropylene tube (with cap), mix briefly
- place tube in a boiling water bath for 10 min (mix tube several times during 10 min incubation)
- add 14 ml 2 M Tris, mix.
- dilute to 10 mg/ml ssDNA by adding 21 ml sterile MQ H_2O (if desired, some 20 mg/ml ssDNA can be saved, and a smaller volume diluted to 10 mg/ml)
- aliquot and store at -20°C

Riboprobe Preparation

Note: It is important to have all reagents at room temperature before addition to reaction. The reaction is set up at room temperature. Also, follow the described sequence of reagent addition. These precautions prevent the linearized DNA from precipitating due to the presence of spermidine.

1. To make a 20 µl transcription labeling reaction:

1.0 µl linearized DNA (1 µg)
3.5 µl sterile H_2O
1.0 µl 10 mM ATP (Final conc = 500 µM)
1.0 µl 10 mM GTP (Final conc = 500 µM)
1.0 µl 10 mM UTP (Final conc = 500 µM)
2.0 µl 100 mM DTT
0.5 µl RNasin (40 units/µl stock)
4.0 µl 5 x transcription buffer
5.0 µl [^{32}P]CTP (200µCi) (Final conc = 12.5 µM)
1.0 µl SP6 or T7 RNA polymerase (15 units/µl stock)

2. Mix reaction, microfuge briefly, and incubate at exactly 40°C for 1 hour in a water bath.

Hint: Just before the 1 h incubation is complete, start preparing Sephadex G-50 spun columns (see below).

3. Remove template DNA:

- add 0.5 µl RNasin
- add 1 µl RQ1 DNase
- mix, microfuge briefly, and incubate at 37°C for 15 min

4. Bring labeling reaction to 100 µl by adding 78.5 µl sterile TE (pH 8.0), mix.

5. Add an equal volume phenol/chloroform/isoamylalcohol, invert repeatedly until emulsified, microfuge 1 min, remove and discard upper aqueous phase.

6. Add an equal volume chloroform/isoamylalcohol, emulsify by inversion, microfuge 1 min, remove and discard upper aqueous phase.

7. Purify reaction (approx. 100 µl) by Sephadex G-50 spun column chromatography.

Sephadex G-50 Spun Column Chromatography

1. Remove plunger (save) from a sterile 1 ml syringe. Insert a small amount (packed height ≈ 2 mm) of baked glass wool into the syringe using baked tweezers. Pack glass wool at bottom of syringe using sterile plunger.

2. Cut the cap off a sterile eppendorf tube (1.5 ml) and place inside (bottom) a 15 ml conical centrifuge tube (not sterile).

3. Place the glass wool-plugged syringe in the conical tube/eppendorf assembly in a manner such that the syringe is "suspended" and its tip just reaches to the inside of the eppendorf tube.

4. Gently swirl bottle of Sephadex G-50/TE to resuspend Sephadex. Remove an aliquot of Sephadex, using a baked pasteur pipet, and transfer it to the "suspended" syringe (ie. insert the pipet tip down to the glass wool pad and slowly withdraw tip as syringe fills with Sephadex). Fill syringe completely with Sephadex slurry.

5. Centrifuge spun column assembly (containing Sephadex) at 3000 rpm (1600 *g*) for 4 min at room temperature.

6. Remove assembly from centrifuge, remove TE from eppendorf tube (using pipet), and again apply Sephadex slurry to fill syringe.

7. Centrifuge spun column assembly at 3000 rpm for 4 min

Note: Final packed volume should be about 1 ml.

8. Apply 100 µl sterile TE to the top of the spun column and centrifuge at 3000 rpm for 4 min.

9. Repeat TE application and centrifugation (ie. step 8).

10. Lift syringe out of 15 ml tube and replace eppendorf tube with a "fresh" de-capped eppendorf tube.

11. Apply labeling reaction (approx. 100 µl) to top of spun column. Centrifuge at 3000 rpm for 4 min.

12. Recover purified probe (approx. 100 µl). Transfer to a fresh eppendorf tube.

13. Count 1 µl of purified probe solution to determine dpm/µl.

    Notes: (1) Place the 1 µl probe solution in 15-20 ml H_2O in a 20 ml scintillation vial. Count Cerenkov radiation. Counting efficiency if approx. 50%.
    (2) Probe specific activity can be estimated using a standard TCA precipitation protocol.

14. Store probe on ice until needed.

Preparation of Prehybridizatioin Solution

To make 20 ml:

Final Conc.	Stock Solution	Volume
60%	100% deion formamide	12 ml
1 ×	20 × SSPE	1 ml
0.5%	10% blotto	1 ml
10%	50% dextran sulphate	4 ml
1%	20% SDS	1 ml
0.5 mg/ml	10 mg/ml ssDNA*	1 ml

* ssDNA is heat denatured before adding to prehybridization solution by placing in boiling water bath for 10 min
- mix prehybridization solution well and preheat to 50°C before adding to membrane

Prehybridization

1. Add prehybridization solution to heat-sealable plastic bag containing membrane with bound RNA.

   Note: Add 0.15-0.25 ml prehybridization solution/cm^2 of membrane.

2. "Roll" bubbles out of bag by placing bag on an angled plate and rolling bag, from bottom to top, with a 10 ml glass pipet.

3. Heat seal bag and place in a tray containing water (50°C). Incubate at 50°C for 1-2 h in a shaking water bath with vigorous agitation.

   Notes: (1) If desired, prehybridization can proceed for much longer than 1-2 h.
   (2) Pyrex brand "Storage Plus" glass trays (blue plastic lids) are very convenient and can be purchased from major department stores (eg. Woodwards). These trays can be baked (not lids) so they are also convenient for gel soaking, membrane soaking, membrane washing, etc.

Preparation of Hybridization Solutiion

Note: During prehybridization, prepare hybridization solution.

To make 20 ml:

Final Conc.	Stock Solution	Volume
60%	100% deionized formamide	11 ml
1 ×	20 × SSPE	1 ml
0.5%	10% blotto	1 ml
10%	50% dextran suphate	4 ml
1%	20% SDS	1 ml

- mix hybridization solution well and preheat to 50°C
- add appropriate volume of purified probe solution (ie. to get 2 × 10^6 dpm/ml hybridization solution) to a solution containing 1 ml ssDNA and 1 ml 100% deionized formamide, mix.
- heat probe/ssDNA/formamide solution at 70°C for 5 min in a water bath, and then add to preheated (50°C) hybridization solution, mix.

Hybridization

1. Dain prehybridization solution from bag containing membrane.

2. Add hybridization/probe solution to bag, roll out bubbles, and then heat-seal bag.

3. Place bag in tray containing H_20 (50°C) and incubate at 50°C with vigorous agitation in a shaking water bath (16-18 h).

Solutions for Membrane Washing

1. 0.2 × SSC

   To make 250 ml:

   - 5.0 ml 10 × SSC
   - bring to 250 ml with sterile MQ H_20

2. 0.2 × SSC/1% SDS

   To make 250 ml:

   - 5.0 ml 10 × SSC
   - 25.0 ml 10% SDS
   - bring to 250 ml with sterile MQ H_20

   Note: This solution is preheated to 70°C before use.

3. 2 × SSC/0.1% SDS

   To make 250 ml:

   - 50 ml 10 × SSC
   - 2.5 ml 10% SDS
   - bring to 250 ml with sterile MQ H_20

4. 2 × SSC

   To make 250 ml:

   - 50 ml 10 × SSC
   - bring to 250 ml with sterile MQ H_2O

Washing Membranes

1. After hybridization is complete, drain hybridization solution from bag.

2. On a pad of paper towels, cut plastic bag around membrane using a scalpel blade. Separate plastic using tweezers, remove membrane (baked tweezers), place in first wash solution (step 3).

3. Briefly rinse membrane in 2 × SSC.

   Notes: (1) Use approx. 250 ml wash solution in tray.
   (2) If desired, 2 membranes can be simultaneously washed in each tray.

4. Wash membrane in 2 × SSC/0.1% SDS (250 ml) vigorously at room temperature for 15 min on a shaker.

5. Wash membrane in preheated (70°C) 0.2 × SSC/1% SDS (250 ml) vigorously at 70°C for 15 min in a shaking water bath.

6. Briefly rinse membrane in 0.2 × SSC.

7. Briefly blot membrane between Whatman 3MM filter paper and then immediately heat-seal membrane in a plastic bag.

   Note: Do not use plastic wrap (eg. Saran Wrap) because it readily generates static artifacts on autoradiograms.

Autoradiography

1. Tape membrane/bag securely into an autoradiographic cassette containing an intensifier screen.

2. In dark room (safelight on), place x-ray film over membrane, securing film to cassette with a single piece of tape.

   Notes: (1) Handle x-ray film with gloved hands only.
   (2) Normally, Kodak XAR-5 film (13 × 18 cm) is used. It is the most sensitive x-ray film available. However, with high intensity hybridization signals, Kodak XRP film provides better band resolution.

3. Using a felt marker, mark on the x-ray film the position of the end (origin) of the membrane.

4. Close cassette and expose film at -70°C. Exposure time depends on intensity of signal, but a 24 h exposure is a good starting point.

X-ray Film Development

1. Remove x-ray film cassette from -70°C and allow to warm to room temperature for 30-60 min before opening in dark.
2. Slowly open cassette (to prevent static artifacts) in dark. Slowly remove film.
3. Develop film for 5 min in Kodak GBX developer (6 min for XRP film). Gently agitate developer for 5 sec each min.
4. Place film in a 1% acetic acid stop bath and continuously agitate for 30 sec.
5. Fix for 2 min in Kodak GBX fixer. Agitate for 5 sec every 30 sec.
6. Rinse film in running tap water for 5-10 min.
7. Allow film to dry (hanging) in a dust free place.

APPENDIX 6: *IN SITU* HYBRIDIZATION

Background

In situ hybridization involves many of the same techniques used for Northern hybridization. It also involves histological techniques such as tissue sectioning, tissue manipulation, and microautoradiography. As with Northern hybridization, the use of riboprobes provides the highest sensitivity in *in situ* hybridization. Several different radioisotopes (eg. ^{125}I, ^{3}H, ^{32}P, and ^{35}S) can be used to label probes for *in situ* hybridization. Non-isotopic labels (eg. avidin-biotin) are also now available, but their use does not result in as high a level of sensitivity as that obtained using radioisotope labels. This protocol has been optimized for use with [^{35}S]-labeled riboprobes. Although [^{3}H]-labeled riboprobes provide higher resolution, their use requires much longer exposures (weeks to months) than that required when using [^{35}S]-labeled riboprobes (days).

General Procedure

Tissue is collected and immediately fixed. It is then cryoprotected and embedded in OTC compound. Embedded tissue can be stored for several months at -70°C.

Day 1 -
- clean and sub microscope slides
- store slides, at least overnight, at 4°C to achieve good tissue adherence to slides.
- slides can be stored at 4°C for several weeks

Day 2 -
- section tissue and thaw-mount onto subbed slides
- tissue mounted on slides can be stored for several days at 4°C before hybridization

Day 3 -
- section pretreatment
- prehybridization
- probe labeling
- start hybridization

Day 4 -
- end hybridization
- wash tissue sections
- start x-ray film (XAR-5) exposure (preliminary autoradiography to estimate signal intensity)

Day 5 -
- develop XAR-5 film
- NTB-2 liquid emulsion tissue coating
- start emulsion drying procedure

Day 6 -
- end emulsion drying procedure
- start emulsion exposure (emulsion is developed at different exposure times, depending on results of preliminary x-ray film exposure.

After emulsion exposure and development, tissue is stained and coverslipped. The tissue is photographed on a microscope using bright and darkfield optics.

Equipment/Materials

- baked glassware for RNA solution preparation
- cryostat
- *in situ* hybridization chambers (Tyler Research Instruments, Edmonton, AB)

- pre-cleaned microscope slides (75 x 25 mm)
- pre-cleaned coverslips (25 x 40 mm)
- slide staining racks and trays (glass; Wheaton)
  Note: Wheaton slide glassware can not be baked. Make RNase free by soaking overnight (or longer) in a dichromate acid bath, followed by extensive rinsing with Milli Q (MQ) H_2O. Store wrapped in aluminum foil.
- Whatman filters (No 2V, 15 cm diameter)
- rubber cement (Lepage's)
- sterile 5 and 10 ml polypropylene tubes with caps
- sterile 50 ml polypropylene centrifuge tubes with caps (Corning)
- pieces of channel iron (approx. 3 feet long, 2 x 1")
- strips of 24 gauge stainless steel (approx. 3 feet long with a 1/8 inch lip folded at 90° along one edge of strip)
- Kodak Nuclear track emulsion NTB-2 (Cat. No. 1654433)
- riboprobe labeling reagents (APPENDIX 5)
- Sephadex G-50 spun column apparatus (APPENDIX 5)
- [^{35}S] Cytidine 5'-(α-Thio) triphosphate (NEN; Cat. No. NEG-064C, 1mCi)
- DDT (Boehringer Manheim; 197 777, 2 g)
- Proteinase K (BM; 745 723, 100 mg)
- RNase A (BM; 109 169, 100 mg)
- tRNA from baker's yeast (BM; 109 495, 100 mg)
- dextran sulphate (Pharmacia; 17-0340-01, 100 g)
- β-mercaptoethanol (Bio-Rad; 161-0710, 25 ml)
- Denhardt's solution (Sigma; D 9905)
- gelatin 275 bloom (Fisher; G8-500, 500 g)
- Permount (Fisher; SP15B-100)
- formaldehyde (Fisher; F79B-500)
- xylene (Fisher; X4-4)
- sodium dihydrogen orthophosphate (BDH; ACS795-34, 500 g)
- EDTA (free acid) (BDH; B10424-32, 250 g)
- sodium acetate trihydrate (BDH; ACS759-40, 2.5 kg)
- chromic potassium sulphate (BDH; B10079-34, 500 g)
- hematoxylin-Harris (without acetic acid) (BDH; RO3312-76)
- PIPES (Sigma; P 9291, 25 g)
- Kodak D-19 developer (Cat. No. 146 4593)
- Kodak fixer (Cat. No. 123-8146)
- glutaraldehyde (Sigma; G5882)
- paraformaldehyde (Fisher)
- Drierite dessicant (Fisher)
- 0.45 and 0.2 μm Nalgene vacuum filters (sterile)
- OCT tissue embedding compound (Miles Scientific)
- isopentane
- liquid nitrogen

Solution Preparation

1. 1 M DTT

   - dissolve 1.54 g DTT in 8 ml sterile MQ H_2O
   - adjust volume to 10 ml with sterile MQ H_2O
   - sterilize by vacuum filtration (0.22 μm)
   - dispense into 1 ml aliquots and store at -20°C

2. tRNA (10 mg/ml)

   - dissolve 100 mg tRNA in 10 ml sterile MQ H_2O
   - dispense into 250 μl aliquots and store at -20°C

3. Proteinase K (20 mg/ml)

- dissolve 100 mg proteinase K in 5 ml sterile MQ H_2O
- dispense into 250 µl aliquots and store at -20°C

4. 20 × PIPES (pH 6.8)

To make 500 ml:

14.62 g EDTA (free acid)
17.12 g PIPES
87.66 g NaCl

- add reagents to approx. 450 ml sterile MQ H_2O. Stir vigorously with a baked magnetic stir bar.
- add 10 N NaOH (approx. 20 ml) to raise pH and assist dissolution.
- when dissolved, adjust pH to 6.8 with more 10 N NaOH
- bring to 500 ml with MQ H_2O
- autoclave 20 min, store at room temperature

5. 100 × Denhardt's Solution

- use Sigma Denhart's lyophilate
- add only 2.5 ml (instead of 5 ml) of sterile MQ H_2O to one vial to make 100 × solution, mix until dissolved
- store in aliquots at -20°C

6. Sheared salmon testes DNA (APPENDIX 5).

7. 20% SDS (APPENDIX 2).

8. Phosphate buffered saline (PBS, pH 7.4)

To make 2000 ml:

19.25 ml 10 N NaOH
33.66 g $NaH_2PO_4.H_2O$

- dissolve reagents in 1900 ml sterile MQ H_2O
- add 8.5 g NaCl, mix until dissolved
- adjust pH to 7.4 with 10 N NaOH
- bring volume to 2000 ml with sterile MQ H_2O
- autoclave 40 min
- store at room temperature

9. 1 M Tris-HCl (pH 7.5)

To make 500 ml:

60.55 g Trizma base

- dissolve Trizma base in 400 ml sterile MQ H_2O
- add 60 ml concentrated HCl
- allow to cool to room temperature
- make final pH adjustment to pH 7.5
- bring to 500 ml with sterile MQ H_2O

- autoclave 20 min
- store at room temperature

10. 5 x TE

To make 2000 ml:

100 ml 1 M Tris-HCl, pH 7.5
20 ml 0.5 M EDTA, pH 8.0 (APPENDIX 2)

- bring to 2000 ml with sterile MQ H_20
- autoclave 40 min
- store at room temperature

11. 3 M Na Acetate (pH 5.2)

To make 1000 ml:

408.1 g Na acetate $3H_20$

- dissolve Na acetate in 800 ml sterile MQ H_20
- adjust to pH 5.2 with glacial acetic acid
- bring to 1000 ml with sterile MQ H_20
- autoclave 20 min
- store at room temperature

12. 70% Ethanol/0.3 M Na acetate

- 1400 ml 100% ethanol
- 200 ml 3 M Na acetate (pH 5.2)
- bring to 2000 ml with sterile MQ H_20

13. 80% Ethanol/0.3 M Na acetate

- 1800 ml 100% ethanol
- 200 ml 3 M Na acetate (pH 5.2)
- bring to 2000 ml with sterile MQ H_20

14. 95% Ethanol

15. 100% Ethanol

16. 10 x SSC (APPENDIX 4)

17. 4 x SSC

18. 2 x SSC

19. 0.1 x SSC

20. 0.5 M NaCl/1 x TE

- 200 ml 5 M NaCl
- 20 ml 1 M Tris-HCl (pH 7.5)
- 4 ml 0.5 M EDTA (pH 8.0)
- bring to 2000 ml with sterile MQ H_20

Solutions for Tissue Fixation and Cryoprotection

1. Solution A

   - 1.42 g Na_2HPO_4 (or 1.78 g $Na_2HPO_4 2H_2O$)
   - dissolve in 50 ml sterile MQ H_2O

2. Solution B

   - 1.56 g $NaH_2PO_4 2H_2O$ (or 1.38 g $NaH_2PO_4 H_2O$
   - dissolve in 50 ml sterile MQ H_2O

3. 10% Paraformaldehyde Solution

   - add 10 g paraformaldehyde to 80 ml sterile MQ H_2O (60°C)
   - mix vigorously at 60°C with a baked magnetic stir bar until mostly dissolved (will still be murky)
   - allow solution to cool to room temperature (still mixing)
   - clear solution by adding (dropwise) 1 N NaOH
   - bring to 100 ml with sterile MQ H_2O

4. 2% Paraformaldehyde/1% Glutaraldehyde Solution

   To make 105 ml:

   32.0 ml Solution A
   20.5 ml Solution B
   21.0 ml 10% paraformaldehyde

   - bring to 105 ml with sterile MQ_2O
   - make final pH adjustment to pH 7.4 with 1 N NaOH
   - prepare this solution the day of tissue collection

5. Phosphate Buffer (pH 7.35)

   To make 1000 ml:

   8.0 g NaCl
   0.2 g KCl
   1.14 g Na_2HPO_4 (anhydrous)
   0.2 g KH_2PO_4

   - dissolve reagents in 950 ml sterile MQ H_2O
   - pH to 7.35 with 1 N NaOH or HCl
   - bring to 1000 ml with sterile MQ H_2O
   - autoclave 20 min
   - store at room temperature

6. Cryoprotection Solution (30% sucrose)

   - 75 g sucrose (Ultrapure; BRL)
   - bring to 250 ml with phosphate buffer

Tissue Collection and Handling

1. Biopsy, dissected, and postmortem tissue are obtained and handled using sterile (or baked) instruments. Handle tissue gently.

2. Immerse tissue pieces (maximum 2 mm^3) in fixative (2% paraformaldehyde/1% glutaraldehyde) for 12 h at 4°C. Use 5-10 ml fixative for each tissue piece.

   Notes: (1) Baked 20 ml glass scintillation vials are convenient for tissue processing.
   (2) Very small tissue pieces require less fixation time.

3. Rinse tissue in three changes of phosphate buffer (pH 7.35), 30 min each change, at 4°C (on ice).

4. Cryoprotect tissue by immersing for 8 h in 30% sucrose solution at 4°C.

   Note: Very small tissue pieces require less cryoprotection time.

Tissue Embedding

1. Fashion small cups (0.5-1 cm diameter) out of aluminum foil (to hold OCT/tissue while freezing).

2. Cool isopentane to approx. -150°C using liquid nitrogen.

   Notes: (1) Use a 500 ml beaker 2/3 full of isopentane.
   (2) Use a thermos to hold the liquid nitrogen that is large enough to fit the 500 ml beaker.
   (3) Fashion heavy gauge wire around neck of 500 ml beaker in a manner such that beaker can be suspended in the N_2 thermos.
   (4) When isopentane just begins to freeze (ie. frozen droplets appear) it is ready for tissue freezing.

3. Place a drop of OCT in a foil cup and position tissue on OCT. Cover tissue with another drop of OCT.

4. Holding foil cup with hemostats, dip in cold isopentane several times until completely frozen.

   Note: Do not allow isopentane to enter foil cup. Also, dip only for 3-5 sec each dip. This procedure prevents OTC/tissue block from cracking.

5. Peel foil cup off frozen block or leave as is. Blocks are stored at -70°C.

   Note: Block should be stored in an air-tight container to prevent dessication of OCT and tissue. Small polypropylene scintillation vials (eg. 7 ml Mini Vials) are convenient for storage; fill excess vial space with OCT and freeze before placing tissue/OCT block in vial.

Microscope Slide Cleaning

1. Cleaning Solution (10% HCl/70% ethanol)

   To make 500 ml:

   - 50 ml concentrated HCl
   - 368 ml 95% ethanol
   - bring to 500 ml with sterile MQ H_2O

2. Submerge slides (in glass slide rack) in acid/ethanol solution (10 min).

3. Submerge slides in sterile MQ H_20 (2 min).

4. Submerge slides in 95% ethanol (2 min).

5. Dry slides in 75°C oven (30 min).

Microscope Slide Coating

1. Heat 500 ml of sterile MQ H_20 to 60°C.

2. Add 2 g gelatin to the 60°C H_20 while mixing with baked magnetic stir bar.

3. When gelatin is completely dissolved, add 0.2 g chromic potassium sulphate and mix until it is dissolved.

4. Filter hot subbing solution through a Whatman No. 2V filter, into an RNase free glass slide tray.

5. Submerge cleaned and dried slides (in a glass slide rack) in hot subbing solution for 15 sec.

6. Dry slides overnight (in rack) at 37°C.

7. Store dry subbed slides at 4°C in a slide box.

Tissue Sectioning and Mounting

1. Transfer frozen (-70°C) tissue/OCT blocks to -20°C cryostat and allow to temperature equilibrate for 30-60 min.

2. Cut sections at 10 µm and thaw-mount onto subbed slides at room temperature (30 min).

   Notes: (1) Mount several sections on each slide.
   (2) Use mounted sections within 1-3 days. Store at 4°C until needed.

Tissue Section Pretreatment

1. Take mounted tissue sections out of 4°C and allow to stand at room temperature for 30 min.

2. Prepare 200 ml of 4% formaldehyde/PBS for each batch (10 slides/glass rack) of slides, and fix slides by immersion (20 min).

   To make 200 ml:

   - 180 ml PBS
   - 20 ml 37% formaldehyde

3. Wash slides in three changes of PBS, 10 min each wash.

4. Deproteinase slides by immersion in 20 µg/ml proteinase K/5 × TE (7.5 min).

To make 200 ml:

- 200 ml 5 × TE
- add 200 µl proteinase K stock (20 mg/ml), mix.

5. Wash slides in two changes of PBS, 15 min each wash.

6. Immerse slides in 4% formaldehyde/PBS for 5 min.

7. Immerse slides in 70% ethanol/0.3 M Na acetate (5 min).

8. Immerse slides in 90% ethanol/0.3 M Na acetate (5 min).

9. Immerse slides in 95% ethanol (5 min).

10. Immerse slides in 100% ethanol (5 min).

11. Allow slides to dry 1 h before starting prehybridization.

Preparation of Prehybridization Solution

Note: The amount of prehybridization solution required to cover tissue sections depends on the size of the sections and the number of sections on a slide. If slides are quite "full" use 500-750 µl prehybridization solution/slide.

1. To make 40 ml:

Stock Solution	Volume
100% deion formamide	20 ml
20 × PIPES	10 ml
100 × Denhardt's	2 ml
20% SDS	0.4 ml
1 M DTT	4 ml
*ssDNA (10 mg/ml)	1 ml
*tRNA (10 mg/ml)	1 ml
sterile MQ H_2O	1.6 ml

* Heat denature for 10 min in a boiling water bath before adding to prehybridization solution.

2. Mix prehybridization solution well. Place in 43°C oven until needed.

Prehybridization

1. Layer 3MM Whatman filter paper on bottom of *in situ* hybridization chamber and soak thoroughly with sterile MQ H_2O. Pre-warm chamber in 43°C oven.

2. Arrange slides with pretreated tissue sections in *in situ* hybridization chamber.

3. Apply prewarmed (43°C) prehybridization solution by carefully pipetting onto tissue sections.

4. Seal chamber and incubate slides at 43°C for 1-3 h.

Riboprobe Labeling with [^{35}S]CTP

1. Set up reaction at room temperature.

2. Reaction (20 µl):

   1.6 µl sterile H_2O
   2.0 µl linearized DNA (2 µg)
   2.0 µl 100 mM DTT
   1.0 µl 10 mM ATP
   1.0 µl 10 mM GTP
   1.0 µl 10 mM UTP
   1.0 µl RNasin (40 units/µl)
   4.0 µl 5 × transcription buffer
   4.4 µl [^{35}S]CTP (314 µCi) [final conc = 12.6 µM)
   2.0 µl SP6 or T7 RNA polymerase (15 units/µl stock)

3. Mix reaction, microfuge briefly, and incubate at exactly 40°C for 1 h in a water bath.

4. Remove template DNA:

   - add 1.0 µl RNasin
   - add 2.0 µl RQ1 DNase
   - mix, microfuge briefly, and incubate at 37°C for 15 min.

5. Bring labeling reaction to 50 µl by adding 23 µl sterile TE (pH 8.0), mix.

6. Extract with an equal volume (50 µl) phenol/chloroform/isoamylalcohol, microfuge 1 min, remove and discard upper aqueous phase.

7. Extract with an equal volume chloroform/isoamylalcohol, microfuge 1 min, remove and discard upper aqueous phase.

Alkaline Hydrolysis of Riboprobes

1. 0.2 M Carbonate Buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3, pH 10.2)

   To make 100 ml:

   0.67 g $NaHCO_3$
   1.27 g Na_2CO_3

   - dissolve in 80 ml sterile MQ H_2O
   - adjust pH to 10.2 with 10 N NaOH
   - bring to 100 ml with sterile MQ H_2O
   - sterilize by vacuum filtration using a 0.2 µm Nalgene filter
   - make just before using or store aliquots at -20°C.

2. 10% Glacial Acetic Acid

   To make 1 ml:

   - 100 µl glacial acetic acid
   - 900 µl sterile MQ H_2O

3. Calculate hydrolysis reaction time:

$$t = \frac{L_o - L_f}{k\ L_o\ L_f}$$

t = time in minutes
L_o = original riboprobe length (kb)
L_f = final riboprobe length (kb)
k = 0.11/kb/min (hydrolysis rate constant)

Note: Hydrolize riboprobes to 100-150 bp to obtain best results.

4. Add an equal volume (50 µl) 0.2 M carbonate buffer to phenol-extracted labeling reaction, mix, and incubate at 60°C for calculated time (from step 3).

5. When 60°C incubation is complete, neutralize reaction by adding 3 µl 3 M Na acetate (pH 5.2) and 5 µl 10% glacial acetic acid, mix.

6. Purify reaction on a Sephadex G-50 spun column (APPENDIX 5), except equilibrate column (twice) with only 50 µl TE. Store purified reaction on ice until needed.

7. Count 1 µl of purified reaction solution:

   - add 1 µl reaction to 5 ml liquid scintillation solution, mix well.
   - count using [^{14}C] program for approx. 96% counting efficiency.

Gel Analysis of Labeled Riboprobes

1. Prepare a 2% agarose denaturing mini-gel:

   1 g agarose
   5 ml 10 x MOPS
   43.5 sterile MQ H_2O

   - dissolve agarose by heating
   - cool gel solution to 60°C, add 2.55 ml 37% formaldehyde, and quickly pour gel
   - allow gel to stand 1 h before using

2. Prepare riboprobe sample(s) for gel loading:

   - dilute 1 µl of riboprobe reaction so that 5 µl = 100,000 dpm.
   - combine 5 µl (100,000 dpm) diluted riboprobe reaction with 15 µl RNA gel loading buffer (APPENDIX 3).
   - mix, microfuge briefly, and heat at 65°C for 10 min.

3. Load sample(s) on gel, run at 100 V for 10 min and then 2-4 h at 30 V.

4. Soak gel in Enlightening autoradiographic enhancer (NEN) for 10 min with agitation.

5. Dry gel on a gel drier or blot overnight between paper towels.

6. Autoradiography/fluorography is performed using XAR-5 or XRP film.

Preparation of Hybridization Solution

To make 10 ml:

Stock Solution	Volume
100% deion formamide	5.0 ml
20 × PIPES	2.5 ml
100 × Denhardt's	0.5 ml
20% SDS	0.1 ml
1 M DTT	1.0 ml
*ssDNA (10 mg/ml)	0.25 ml
*tRNA (10 mg/ml)	0.25 ml
dextran sulphate	1 g (final conc = 10%)

* Heat denature for 10 min in a boiling water bath before adding to hybridization solution.

- mix solution well by shaking at 43°C, to dissolve dextran sulphate.

Hybridization

1. Add alkaline hydrolized riboprobe to an appropriate volume of hybridization solution (43°C) to make hybridization solution 1.0×10^7 dpm/100 µl.

2. Drain prehybridization solution from tissues by placing slides in vertical position (in *in situ* hybridization chamber) for 10-20 sec.

3. Apply hybridization solution containing riboprobe by carefully pipetting on tissue sections.

   Note: The amount of hybridization solution required varies depending on number of sections on a slide and section size. Use 50-100 µl on a "full" slide.

4. Carefully place a coverslip on each slide and seal onto slide using rubber cement.

   Note: Dispense rubber cement from a 10 ml syringe using an 18 gauge needle. If rubber cement is difficult to dispense, dilute with a few drops of ethyl acetate.

5. Replace *in situ* hybridization chamber into oven and incubate at 43°C for 16-18 h.

Tissue Section Washing

Note: Perform washes in glass slide racks (10 slides/rack) and trays, using 200 ml wash solution in each tray.

1. Remove hybridization chamber from 43°C oven and allow to stand at room temperature for 1 h before starting wash (prevents section loss).

2. To remove coverslips:

   - remove rubber cement using tweezers.

- immerse slides (individually) in 4 × SSC/14 mM β-mercaptoethanol until coverslips "float" off (3-5 min at room temperature).

To make 1000 ml 4 × SSC/14 mM β-mercaptoethanol:
- add 1 ml β-mercaptoethanol stock solution (14 M) to 1000 ml 4 × SSC.

3. Wash slides in three changes 4 × SSC/14 mM β-mercaptoethanol, 10 min each change (room temperature).

4. Wash slides in three changes 4 × SSC, 10 min each change (room temperature).

5. RNase slides at 37°C (30 min).

To make 200 ml RNase solution:

- add 200 µl RNase stock solution (10 mg/ml) to 200 ml of prewarmed (37°C) 0.5 M NaCl/1 × TE solution, mix.

6. Wash slides in prewarmed (37°C) 0.5 M NaCl/1 × TE (no RNase) at 37°C (30 min).

7. Wash slides in 2 × SSC at room temperature (15 min).

8. Wash slides in prewarmed (43°C) 0.1 × SSC at 43°C (45 min).

9. Air dry slides for at least 3 h.

10. Expose slides (48 h) to x-ray film (XAR-5) to estimate signal intensity and appropriate exposure time for emulsion autoradiography.

Liquid Emulsion Autoradiography

Note: A very clean, light-tight darkroom and careful attention to detail are critical to the generation of quality emulsion autoradiograms. All emulsion handling, slide coating and handling, and development procedures are performed in the dark. However, it is necessary to check for the absence of bubbles on emulsion-coated test slides using a safelight (Kodak No. 2 filter, 15W bulb). Work at a minimum distance of four feet from the safelight if it is needed at any other time. Wet emulsion is least sensitive to light (ie. avoid using safelight after emulsion has dried on slides).

Set-up in Darkroom

1. Water bath at 44°C containing rack to accomodate 50 ml tube(s). Alternatively, place a 500 ml beaker containing 300 ml H_2O in water bath to serve as a rack.

2. Large shallow tray to accomodate pieces of channel iron. Place ice in a large plastic bag and flatten bag in tray. Lay channel iron on bag of ice to keep cooled.

3. NTB-2 emulsion, clean blank slides, aluminum foil, slide boxes, large dessicators containing dessicant (to accomodate slide boxes), stainless steel strips, light-tight cupboard or box to accomodate stainless steel strips, Kimwipes, extra slide boxes, and hybridized slides.

4. Transfer 25 ml H_20 (HPLC grade) to a 50 ml tube with cap and place in 44°C water bath.

Emulsion Coating Slides (In Dark)

1. Place NTB-2 in 44°C water bath to melt (45 min).

   Note: Place NTB-2 container in a 500 ml beaker containing 200 ml H_20.

2. Pour 25 ml melted emulsion into 50 ml tube containing prewarmed 25 ml HPLC H_20. Slowly stir diluted emulsion with a blank slide. Replace tube in water bath and allow bubbles to rise (15 min).

3. Dip several blank slides and examine for the presence of bubbles. Use safelight only briefly and at maximum possible distance from emulsion stock. Continue to dip blank slides until no bubbles are observed on slides.

4. Place stainless steel strips on channel iron pieces to cool. Just before starting to dip a slide series, wipe excess condensation from cooled strips with tissue paper (Kimwipes).

5. Have slides arranged in boxes such that order of slides in known throughout slide handling procedure. Dip sample slides into emulsion one at a time; dip slide into emulsion to completely cover tissue and hold for 2 sec. Withdraw slide slowly and blot (5 sec) slide edge on Whatman 3MM filter paper to drain excess emulsion. Carefully wipe back of slide with tissue (Kimwipe). Place slide (horizontal) on cooled stainless steel strip (in predetermined order).

6. After a series of 15 slides have been placed on cooled strip, allow emulsion to gel for 10 min.

7. Carefully lift strip containing slides from channel iron and place in light-tight cupboard or box. Allow emulsion to dry for 2 h (ie. room temperature and humidity).

8. Arrange slides in a slide box and place in a large dessicator (no lids on slide boxes). Wrap dessicator with foil and place in light-tight cupboard or box. Allow slides to dry for another 10-12 h.

9. Place a package of dessicant in each slide box. Close box and wrap in several layers of foil. Do not seal box with tape. Label boxes appropriately.

   Note: Package dessicant in Kimwipe tissue paper and place in one end of slide box. Use a blank slide to separate package from coated slides.

10. Seal boxes in plastic bags containing another, larger package of dessicant. Expose slides at 4°C.

Development of Emulsion (In Dark)

1. Precool D-19 developer, MQ H_20 (stop bath), and fixer to 15°C (59°F) in slide trays.

2. Remove slides from 4°C and allow to warm at room temperature (in closed box) for 30 min before developing.

3. Arrange slides in slide racks and develop in D-19 for 2 min. Gently agitate rack every 30 sec.

4. Rinse slides in MQ H_20 for 15 sec using gentle agitation.

5. Fix slides in Kodak fixer for 5 min. Gently agitate rack once every minute.

6. Rinse slides in running tap water (15°C) for 30 min. This step is performed in a large glass tray.

Tissue Staining (hematoxylin)

1. Stain tissue in filtered hematoxylin (1 min)

   Note: Staining procedure is performed using solutions at room temperature.

2. Rinse with MQ H_20 (5 sec).

3. Dehydrate using 30, 60, 80, 95, 100, and 100% ethanol (30 sec each).

4. Clear in two changes of xylene, 30 sec each change. Use fresh xylene changes for each set of 10 slides.

5. Blot excess xylene, apply 1-3 drops of Permount, and coverslip. Dry slides on a slide warmer (40°C) or leave overnight.

6. Scrape emulsion from slide back using a razor blade. Remove excess Permount using xylene.

Photomicrography

Use light and darkfield optics. Record images using color slide film (Kodak Ektachrome 200 ASA) and black and white negative film (Ilford HP-5 400 ASA). Lightfield exposures are as indicated by microscope exposure meter. Darkfield exposures are determined by bracketing test exposures at 1/2 stops below the indicated exposure. Start at one to two full stops below indicated exposure.

Importance of Specificity Controls

There are several specificity controls that must be performed along with *in situ* hybridization experiments. The importance of controls can not be overemphasized. Detailed discussions of controls for *in situ* hybridization have been presented (Coghlan *et al.* Anal. Biochem. 149:1, 1985; Tecott *et al.* Ch. 6 in In Situ Hybridization: Applications to Neurobiology. Oxford, NY, 1987).